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| (54) Title: TAXOL PRODUCTION VIA GENERATION OF EXTRACHROMOSOMAL DNAs IN THE FUNGUS <i>PESTALOTIOPSIS</i> | | | |
| (57) Abstract <p>This invention relates to compositions and methods useful for producing extrachromosomal DNA, wherein such extrachromosomal DNA codes for a product which changes the level of taxol production. More specifically, the present invention relates to such compositions and methods for producing extrachromosomal DNA based on the unexpected discovery that the <i>Pestalotiopsis</i> fungi add one or more telomeric repeats to the ends of exogenous DNA.</p> | | | |

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**TAXOL PRODUCTION VIA GENERATION OF EXTRACHROMOSOMAL DNAs
IN THE FUNGUS *PESTALOTIOPSIS***

5 **CROSS REFERENCES TO RELATED APPLICATIONS**

This application claims priority to U.S. Provisional Patent Application No. 60/ 091,667, filed on July 2, 1998, which is herein incorporated by reference in its entirety.

10 This application is also related to U.S. Provisional Patent Application No. 60/091,668, filed on July 2, 1998 and to co-pending application Attorney Docket No. 47714-5001-WO, filed on July 2, 1999, which are both herein incorporated by reference in their entireties.

15 **STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY
SPONSORED RESEARCH**

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FIELD OF THE INVENTION

The present invention pertains, in general, to the generation of extrachromosomal DNAs. In particular, the present invention pertains to extrachromosomal DNAs produced by introducing DNA into a *Pestalotiopsis* sp. cell, wherein such introduced DNA codes
25 for a product which changes the level of taxol production.

BACKGROUND OF THE INVENTION

30 All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

***De novo* Addition of Telomeric or Telomerelike Sequences**

De novo addition of telomeric or telomerelike sequences to exogenous DNA has been reported in several taxonomically diverse species.

5 In the ciliated protozoan *Paramecium tetraurelia*, microinjection of supercoiled DNA into the macronucleus results in apparent random linearization and the addition of paramecium-type telomere sequences to the ends of the DNA (Gilley *et al.*, 1988). The introduced linear DNA is directly modified by the addition of telomeric repeats and the resultant molecules replicate efficiently.

10 Telomeric sequences were also added *de novo* to linearized *Cryptococcus neoformans* transforming plasmids. *C. neoformans* is a basidiomycete with the teleomorph *Filobasidiella neoformans*. The added telomeric sequences are simple repeats of the octanucleotide AGGGGGTT (Edman *et al.*, 1992). Recovered plasmids showed increased transformation efficiencies in the supercoiled state (up to 200 transformants per μ g) and in the linear state (up to 90,000 transformant per μ g). While vectors derived from
15 the plasmids produced via this process should prove useful for introducing genes back into *C. neoformans* (see, e.g., Varma *et al.*, 1994), the fact that this organism is a heterothallic basidiomycetous yeast that causes meningeal and systemic infections in humans, its utility for all applied genetic uses will be limited.

20 The fungal rearrangement of normally integrative plasmids results in the creation of linear self-replicating plasmids in *Fusarium oxysporum* (Powell and Kistler, 1990). The rearrangement results in the addition of fungal DNA, including the telomere sequence TTAGGG, to plasmid termini at a frequency much lower than in *Cryptococcus* and *Pestalotiopsis*. Unlike the system discovered for *C. neoformans*, the linear
25 extrachromosomal DNA in *F. oxysporum* undergoes partial duplication of the transforming DNA. A DNA sequence containing the repeated telomeric sequence flanked by a region of twofold symmetry consisting primarily of pUC12 DNA was shown to produce autonomous replication and enhance transformation in several species at relatively low frequencies. For example, a transformation of approximately 300-3,000
30 transformants per μ g of vector was obtained for *F. oxysporum* and 2,000 transformant per μ g were obtained for the related fungus *Nectria haematococca*. The rate of

transformation using the circular form of a new linear plasmid has yielded 5,600 transformants per μg of DNA (Garcia-Pedrajas, *et al.*, 1996). *F. oxysporum* has the following taxonomic lineage: Eukaryota; Fungi; Ascomycota; Euascomycetes; Pyrenomycetes; Hypocreales; Nectriaceae; mitosporic Nectriaceae; and Fusarium (National Center for Biotechnology Information (NCBI) Taxonomy Database).

In vivo modification of transforming DNA introduced into *Histoplasma capsulatum* included duplication of the plasmid sequence and teleomeric addition at the termini of linear DNA (Woods *et al.*, 1992). *H. capsulatum*, having the teleomorph *Ajellomyces capsulatus*, has the following taxonomic lineage: Eukaryota; Fungi; Ascomycota; Euascomycetes; Plectomycetes; Onygenales; Onygenaseae; and Ajellomyces (National Center for Biotechnology Information (NCBI) Taxonomy Database). Similar to the situation with *C. neoformans* discussed above, the fact that *Histoplasma capsulatum* is a dimorphic pathogenic fungus that is a major cause of respiratory and systemic mycosis in mammals will limit its utility in mammalian research.

As summarized above, the *de novo* addition of telomeric sequences to exogenous DNA has been previously reported in the taxonomically diverse species *Paramecium tetraurelia*, *Cryptococcus neoformans*, *Fusarium oxysporum* and *Histoplasma capsulatum*. The actual *de novo* process which occurs in each of these organisms differs in various ways. For example, the plasmids produced in *P. tetraurelia* and *C. neoformans* are approximately the same size or smaller than the input DNA, while the plasmids are larger in *F. oxysporum* and *H. capsulatum*, due at least in part to duplication of the input sequences. Some of the particular aspects of these processes limit their usefulness for genetic studies or mammalian transformation. For example, each of these organisms either adds telomeric sequences which are not useful for mammalian transformation (*P. tetraurelia*, *C. neoformans*), are dangerous mammalian pathogens (*C. neoformans*, *H. capsulatum*), or add the telomeric repeats at a relatively low efficiency (*F. oxysporum*). Thus, there currently exists a need for an improved, safe process for the *de novo* production of extrachromosomal DNA with terminal telomeres, especially for the production of extrachromosomal DNA useful for the transformation of animals.

Pestalotiopsis microspora

Pestalotiopsis microspora is a filamentous fungus that lives as an endophyte in the

inner bark of certain trees, including the Himalayan yew tree (*Taxus wallachiana*) (Strobel *et al.*, 1996); bald cypress (*Taxodium distichum*) (Li *et al.*, 1996); and *Torreya taxifolia*, a rare tree with a close taxonomic relationship with *Taxus brevifolia* (Lee *et al.*, 1995). *P. microspora* has an endophytic-pathologic relationship with *T. taxifolia*. While the fungus can reside in the inner bark of symptomless trees, certain physiological and/or environmental factors appear to trigger the fungus into producing the phytotoxins pestalopyrone, hydroxypestalopyrone, and pestaloside.

Both *P. microspora* and its yew tree hosts produce taxol, a diterpenoid secondary metabolite with demonstrated efficacy against certain human cancers (Georg *et al.*, 1994). Cultures of *Pestalotiopsis* sp. have also been shown to produce a number of different compounds in addition to taxol, including a glucoamylase which digests starch (U.S. Patent Number 5,604,128), non-peptidic endothelian antagonists (Ogawa *et al.*, 1995), a highly branched galactomannan with anti-diabetic activity (Kiho *et al.*, 1997); and mycarosyl macrolide antibiotics (U.S. Patent Number 3,784,447).

Although a teleomorph of *P. microspora* has not been observed, several other species within the *Pestalotiopsis* genus form ascospores under appropriate conditions (Nag Raj, 1993).

Known isolates of various *Pestalotiopsis* species include *P. funerea* IFO 5427 (SANK 15174); *P. microspora* IFO 31056 and CP-4; *P. asaciae* IFO 31054; *P. crassiusla* IFO 31055; *P. neglecta* (SANK 13390; FERM BP-3501); and *P. royenae* (ATCC11816) (see, e.g., U.S. Patent Number 3,784,447; U.S. Patent Number 5,604,128; and Li *et al.*, 1996). Isolates are available from the following depositories as indicated by the deposit numbers associated with each isolate: (1) IFO = Institute for Fermentation in Osaka, Japan; (2) FERM = Fermentation Research Institute, Agency of Industrial Science and Technology, Japan; and (3) ATCC = American Type Culture Collection, United States.

Taxol

The group of complex terpene-type compounds known as taxanes and taxane-related compounds have proven to have important anti-cancer properties. Examples of such compounds include taxol, baccatin and cephalomannine.

Taxol, whose generic name as a drug is paclitaxel, is found in the stem bark of the Western or Pacific Yew, *Taxus brevifolia*, as well as in *T. baccata*, *T. cuspidata* and all

other yew species. The Western or Pacific yew is a slow growing conifer found in the under story of old growth stands in the Pacific Northwest. Ten thousand kilograms of bark are required to produce one kilogram of taxol, which is enough to treat only 500 patients. For this reason the chemical synthesis of taxol has aroused great interest. However, the sterically crowded, chemically sensitive and chirally complex taxane ring structure has essentially forestalled any practical *de novo* synthesis. As a result, current chemical efforts are focused on semisynthesis from more readily available congeners. The chemistry of taxol and related diterpenoids has been described in two excellent recent review articles (Kingston, 1991; Nicolaou *et al.*, 1994), the entire disclosures of which are incorporated herein by reference.

Taxol is a naturally occurring diterpenoid which has great potential as an anti-cancer drug, and which has shown activity in several tumor systems. Taxol was first isolated and its structure reported by Wani *et al.* (1971). Taxol is currently on the market. It had demonstrated efficacy with manageable side effects in 30 to 35% of cases of ovarian cancer and 56% of cases of metastatic breast cancer.

The biological activity of taxol is related to its effect on cell division. Microtubules form during the mitotic spindle during cell division. Taxol interferes with depolymerization of the tubulin forming the microtubules of the mitotic spindle, which is essential for cell division to take place. Thus, taxol causes cell division to stop. The mechanism of action of taxol is unique since it promotes the formation of tubulin polymers, whereas other anti-cancer drugs, such as vinblastine and vineristine, prevent microtubule formation.

Although no high yielding source of taxol has been found, a related compound baccatin is much more readily available from the needles of the European yew, *Taxus baccata*. This has led to a very active exploration of semi-synthetic routes from baccatin to taxol.

Pestalotiopsis microspora isolated from the bark, phloem and/or xylem of the Himalayan yew (*Taxus wallachiana*) and the bald cypress (*Taxodium distichum*) produces taxol in significant but not commercially viable amounts (Strobel *et al.*, 1996; Li *et al.*, 1996)). The taxol biosynthetic pathway in yew plants is beginning to be elucidated (Koepp *et al.*, 1995; Wildung and Croteau, 1996), but in fungi it is completely unknown.

Studies of the taxol biosynthetic pathway in the filamentous fungus *P. microspora* had been undertaken by the present inventors for two primary reasons. First, such knowledge will permit rational molecular genetic manipulation of *P. microspora* so that commercially meaningful quantities of taxol may be produced by this easily culturable organism. A number of filamentous fungi have been modified genetically to enhance their commercial utility (Upshall *et al.*, 1991). Second, knowledge of the pathway and gene sequences may indicate if taxol biosynthesis evolved independently, was present in the most recent common ancestor, or is the result of genetic exchange between the fungus and its yew host. Many other examples of the production of potent bioactive metabolites by both fungi and plants are known (Isaac, 1992; Gay *et al.*, 1994) but the evolutionary and genetic bases for this are not understood.

While developing the necessary molecular genetic techniques to elucidate the fungal taxol biosynthetic pathway, the inventors of the present invention transformed *P. microspora* with a plasmid containing the bacterial hygromycin resistance gene controlled by an *Aspergillus* promoter and terminator. Although the same general methods used to accomplish this transformation have resulted in chromosomal integration in the vast majority of fungi (Lemke and Peng, 1995), unexpectedly, the majority of *P. microspora* transformants contained extrachromosomal DNAs with terminal telomeric repeats added *in vivo*. We herein describe the *in vivo* addition of terminal telomeric repeats to transforming DNA in *P. microspora*, the properties of the extrachromosomal DNAs formed by this modification, as well as the use of such a process in genetic research and for the production of transgenic organisms, including transgenic mammals. In particular, we herein describe methods of producing taxol based on our surprising discovery that *P. microspora* produces extrachromosomal DNA from DNA introduced into its cells.

SUMMARY OF THE INVENTION

This invention comprises compositions and methods useful for producing extrachromosomal DNA. More specifically, the present invention provides compositions and methods based on the unexpected discovery that the *Pestalotiopsis* fungi add one or more telomeric repeats to the ends of exogenous DNA.

This invention provides methods of adding one or more telomeric repeats to

exogenous DNA by introducing exogenous DNA into a *Pestalotiopsis* cell. The exogenous DNA can consist of telomeric or non-telomeric DNA. Furthermore, the exogenous DNA can be either circular, linear or multimeric DNA. Preferably, the exogenous DNA codes for a product which changes the level of taxol production, such as one or more enzymes or enzyme subunits responsible for taxol biosynthesis. Thus, the present invention provides methods of increasing taxol production on a cellular, tissue, organ or whole-organism basis.

This invention also provides methods of generating extrachromosomal DNA by introducing exogenous DNA into a *Pestalotiopsis* cell, wherein such DNA codes for a product which changes the level of taxanes, such as DNA which codes for taxol biosynthesis. Extrachromosomal DNA produced using the procedures of this invention can be isolated and used to transform a prokaryotic or eukaryotic cell. Thus, the extrachromosomal DNAs produced by the procedures of this invention can be used to transform a *Pestalotiopsis* cell or other eukaryotic cell with one or more genes coding for an enzymatic step in taxane biosynthesis, including the production of taxol and related diterpenoids.

This invention further provides methods of generating a replicable nucleic acid element by introducing exogenous DNA into a *Pestalotiopsis* cell, wherein the exogenous DNA codes for a product which changes the level of taxol production, such as enzymes or enzyme subunits which catalyze one or more steps in taxol biosynthesis. Therefore, the procedures of the present invention can be used to transform a *Pestalotiopsis* cell with genes related to cellular taxol production. Alternatively, the replicable nucleic acid elements can be isolated and used to transform other eukaryotic cells with genes related to cellular taxol production.

This invention also provides methods of adding one or more telomeric repeats to exogenous DNA wherein the method comprises introducing the exogenous DNA into a cell of an ascomycete, with the proviso that the ascomycete is not *Fusarium oxysporum*.

One particular embodiment of the present invention comprises introducing exogenous DNA into a *Pestalotiopsis* cell; permitting one or more telomeric repeats to be added to the exogenous DNA to produce extrachromosomal DNA; extracting the extrachromosomal DNA from the transformed *Pestalotiopsis* cell; and introducing the

extracted extrachromosomal DNA into a second cell. The exogenous DNA utilized in the invention can be either from any *Pestalotiopsis* sp. or be non-*Pestalotiopsis* DNA. The second cell can be either a different *Pestalotiopsis* cell or other eukaryotic cell. DNA of particular interest is DNA which codes for a product which changes the level of taxol production.

More specifically, the present invention involves introducing exogenous DNA coding for enzymes or enzyme subunits involved in taxol biosynthesis into a *Pestalotiopsis* cell and thereby increasing production of taxol by the transformed *Pestalotiopsis* cell. Alternatively, the present invention involves introducing exogenous DNA coding for enzymes or enzyme subunits involved in taxol biosynthesis into a *Pestalotiopsis* cell, extracting the extrachromosomal DNA's produced by such an method, and then introducing the extracted extrachromosomal DNA's into a yew plant cell, thereby increasing production of taxol by the transformed yew cells. The taxol-related gene(s) can be isolated from either *Pestalotiopsis* or a tree species which produces taxol, such as the Pacific yew. Examples of genes coding for taxol biosynthesis include those coding for the enzymes or enzyme subunits responsible for the cyclization of the universal diterpenoid precursor geranylgeranyl diphosphate to taxa-4(5),11(12)-diene; the cytochrome P450-catalyzed hydroxylation of this olefin to taxa-4(20),11(12)-dien-5 alpha-ol; and the acetyl CoA-dependent conversion of the alcohol to the corresponding acetate ester. Examples of particular DNA sequences useful in the present invention include those coding for taxadiene synthase, taxadiene-5-hydroxylase, acetyl-coenzyme A, or those coding for any one of the additional oxidation steps in taxol biosynthesis.

The present invention also provides telomerase enzymes or enzyme subunits isolated and purified from *Pestalotiopsis*; DNA sequences coding for such telomerase enzymes or enzyme subunits; and a recombinant DNA construct comprising the RNA sequence of such telomerase enzymes or enzyme subunits.

The present invention also provides methods of producing stable DNA fragments by adding one or more telomeric repeats to the ends of the DNA using telomerase isolated from *Pestalotiopsis*.

The present invention further provides artificial chromosomes comprising the DNA sequences of a centromere, an autonomous replication sequence, a selectable marker

and a sequence coding for a *Pestalotiopsis* telomerase. The artificial chromosomes of the present invention can further comprise additional genes of interest, wherein those additional genes code for one or more enzymes or enzyme subunits responsible for taxol biosynthesis. Particular embodiments of the present invention provide artificial
5 chromosomes which include one or more genes coding for taxadiene synthase, taxadiene-5-hydroxylase and acetyl-coenzyme A.

The present invention also provides host cells transformed with a recombinant nucleic acid comprising an oligonucleotide having a contiguous sequence of at least 25 nucleotides in a sequence complementary or identical to a *Pestalotiopsis* genomic DNA
10 sequence encoding the RNA component of *Pestalotiopsis* telomerase. The recombinant nucleic acid can further comprise a promoter positioned to drive the transcription of an RNA having a sequence complementary to the oligonucleotide. The host cells utilized in this aspect of the invention include eukaryotic cells other than those of *Pestalotiopsis*.

The present invention also provides methods for producing the RNA component of
15 *Pestalotiopsis* telomerase comprising the step of culturing a prokaryotic or a eukaryotic host cell transformed with a recombinant nucleic acid comprising a promoter positioned to drive the transcription of a DNA sequence encoding an RNA component of *Pestalotiopsis* telomerase.

The invention also provides methods for producing a recombinant *Pestalotiopsis*
20 telomerase enzyme, said method comprising transforming a prokaryotic or a eukaryotic host cell capable of expressing protein components of telomerase, with a recombinant nucleic acid comprising a promoter positioned to drive the transcription of a DNA sequence encoding the RNA component of *Pestalotiopsis* telomerase, said recombinant nucleic acid functioning to produce the oligonucleotide in the host cell, and culturing said
25 host cells transformed with said vector under conditions such that the protein components and RNA component are expressed and assemble to form an active telomerase molecule capable of adding sequences to telomeres of chromosomal DNA.

This invention also provides the RNA component of, as well as the gene for the RNA component of, *P. microspora* telomerase in substantially pure form, as well as
30 nucleic acids comprising all or at least a useful portion of the nucleotide sequence of the RNA component of *P. microspora* telomerase. The present invention also provides RNA

component nucleic acids from other species, which nucleic acids share substantial homology with the RNA component of *P. microspora* telomerase.

Other useful nucleic acids of the invention include nucleic acids with sequences complementary to the RNA component; nucleic acids with sequences related to but
5 distinct from nucleotide sequences of the RNA component and which interact with the RNA component or the gene for the RNA component or the protein components of *P. microspora* telomerase in a useful way; and nucleic acids that do not share significant sequence homology or complementarity to the RNA component or the gene for the RNA component but act on the RNA component in a desired and useful way.

10 Thus, one type of useful nucleic acid of the invention is an antisense oligonucleotide, a triple helix-forming oligonucleotide, or other oligonucleotide that can be used *in vivo* or *in vitro* to inhibit the activity of *P. microspora* telomerase. Such oligonucleotides can block telomerase activity in a number of ways, including by preventing transcription of the telomerase gene (for instance, by triple helix formation) or
15 by binding to the RNA component of telomerase in a manner that prevents a functional ribonucleoprotein telomerase from assembling or prevents the RNA component, once assembled into the telomerase enzyme complex, from serving as a template for telomeric DNA synthesis. Typically, and depending on mode of action, these oligonucleotides of the invention comprise a specific sequence of from about 10 to about 25 to 200 or more
20 nucleotides that is either identical or complementary to a specific sequence of nucleotides in the RNA component of telomerase or the gene for the RNA component of telomerase.

Another type of useful nucleic acid of the invention is a ribozyme able to cleave specifically the RNA component of *P. microspora* telomerase, rendering the enzyme or enzyme subunits inactive. Yet another type of useful nucleic acid of the invention is a
25 probe or primer that binds specifically to the RNA component of *P. microspora* telomerase and so can be used, *e.g.*, to detect the presence of *P. microspora* telomerase in a sample. Finally, useful nucleic acids of the invention include recombinant expression plasmids for producing the nucleic acids of the invention. One especially useful type of such a plasmid is a plasmid used for genetic transformation of animals.

30 In another aspect, the invention provides methods for treating a condition associated with the telomerase activity within a cell or group of cells by contacting the

cell(s) with a therapeutically effective amount of an agent that alters telomerase activity in that cell. Such agents include the telomerase RNA component-encoding nucleic acids, triple helix-forming oligonucleotides, antisense oligonucleotides, plasmids, ribozymes, small molecules, other chemical entities. In a related aspect, the invention provides
5 pharmaceutical compositions comprising these therapeutic agents together with a pharmaceutically acceptable carrier or salt.

In yet another aspect, the invention provides diagnostic methods for determining the level, amount, or presence of the RNA component of *P. microspora* telomerase, telomerase, or telomerase activity in a cell, cell population, or tissue sample, or an extract
10 of any of the foregoing. In a related aspect, the present invention provides useful reagents for such methods (including the primers and probes noted above), optionally packaged into kit form together with instructions for using the kit to practice the diagnostic method.

In still another aspect, the present invention provides recombinant *P. microspora* telomerase preparations and methods for producing such preparations. Thus, the present
15 invention provides a recombinant *P. microspora* telomerase that comprises the protein components of *P. microspora* telomerase as well as the protein components of telomerase from a species with an RNA component substantially homologous to the RNA component of *P. microspora* telomerase in association with a recombinant RNA component of the invention. Such recombinant RNA component molecules of the invention include those
20 that differ from naturally occurring RNA component molecules by one or more base substitutions, deletions, or insertions, as well as RNA component molecules identical to a naturally occurring RNA component molecule that are produced in recombinant host cells. The method for producing such recombinant telomerase molecules comprises transforming a prokaryotic or eukaryotic host cell that expresses the protein components
25 of telomerase with a recombinant expression vector that encodes an RNA component molecule of the invention, and culturing said host cells transformed with said vector under conditions such that the protein components and RNA component are expressed and assemble to form an active *P. microspora* telomerase molecule capable of adding sequences (not necessarily the same sequence added by native telomerase) to telomeres of
30 chromosomal DNA.

In another aspect, the invention provides methods for purifying the protein

components of *P. microspora* telomerase as well as the protein components of telomerase from a different species with an RNA component substantially homologous to the RNA component of the *Pestalotiopsis* telomerase. The present invention also provides methods for isolating and identifying nucleic acids encoding such protein components. In related aspects, the present invention provides purified *P. microspora* telomerase and purified telomerase of species with an RNA component substantially homologous to the RNA component of *P. microspora* telomerase, as well as purified nucleic acids that encode one or more components of such telomerase preparations. The present invention also provides pharmaceutical compositions comprising as an active ingredient the protein components of *P. microspora* telomerase or a nucleic acid that encodes or interacts with a nucleic acid that encodes a protein component of *P. microspora* telomerase.

One skilled in the art can easily make any necessary adjustments in accordance with the necessities of the particular situation.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Southern blot of undigested total DNA from *P. microspora* wild-type and transformants. Lane 33, 200 pg each of linear (Lin) and circular (Cir) pDH33. Lane Wt, wild-type total DNA. Lanes 1 through 8, transformant DNA. The position of chromosomal DNA is indicated (Chm, approximately 50 kb). Numbers to the right are molecular size markers (in kb). For lanes 1 through 8, 1.5 µg of DNA was electrophoresed through 0.6% agarose, transferred to nylon membrane, and hybridized with ³²P-labeled random primed pDH33.

Figure 2. a. Stability of the hygromycin resistant phenotype of *P. microspora* transformants in the absence of selection. After the indicated number of days of growth on PDA, transformants were transferred to PDA containing 200 µg/ml hygromycin and colony areas were measured after 7 days (see Materials and Methods). ■, tr14 and □, tr19, are integrants. ▼, tr10; ○, tr13; ▲, tr15; and ◆, tr17, are transformants with extrachromosomal DNAs.

- b. The effect of hygromycin concentration on the vegetative growth of *P. microspora* wild-type and transformants. Colony areas were measured after 7 days growth on PDA containing the indicated concentrations of hygromycin. ●, wild-type. ■, tr14 and □, tr19, integrants. ▼, tr10; ○, tr13; ▲, tr15; and ◆, tr17, transformants with extrachromosomal DNAs.

- Figure 3.** a. The stability of *P. microspora* transformant extrachromosomal DNAs with growth in the presence and absence of selection. Lane 33, 200 pg each of linear (Lin) and circular (Cir) pDH33. Lane Wt, wild-type *P. microspora* total DNA. Lanes 1, 3, 6, and 9, total DNA from transformants before growth (stored at 4°C). Lanes 4, 7, and 10, total DNA from transformants after 6 months growth under selection (PDA with 200 µg/ml hygromycin). Lanes 2, 5, 8, and 11, transformant total DNA after 35 days growth without selection (PDA without hygromycin). Numbers to the right are molecular size markers (in kb) and Chm is the position of chromosomal DNA (50 kb). 1.5 µg of DNA was electrophoresed through 0.6% agarose, transferred to nylon membrane, and hybridized with ³²P-labeled random primed pDH33.
- b. Restriction digests of total DNA from tr10 before (lanes marked 0) and after (lanes marked 6) 6 months growth under selection (PDA with 200 µg/ml hygromycin). Numbers to the right are sizes of restriction fragments (kb). Gel conditions were as in Figure 3a.

- Figure 4.** a. PCR amplification of *P. microspora* transformant tr3 total DNA. Lane Wt, amplification of wild-type total DNA with primer TE2 yielded no visible product. Lane 1, amplification of tr3 total DNA with primer TE2 yielded full-length 5.5 kb extrachromosomal DNA. Lane 2, amplification of tr3 total DNA with primers TE2 and Hyg2 yielded the 2.4 kb 3' fragment of extrachromosomal DNA. Lane 3, amplification of tr3 total DNA with primers TE2 and Hyg1 yielded the 3.1 kb 5' fragment of extrachromosomal DNA. Lanes 4 and 5, *Ban*I restricted PCR product from lane 1 and pDH33, respectively. Numbers to the right are molecular size markers.
- b. Structure of the transformant tr3 PCR amplified 5.5 kb

extrachromosomal DNA. Restriction sites shown are common to the amplified DNA and pDH33. Arrows indicate primers used for PCR.

c. Nucleotide sequences at the pDH33-telomeric repeat junctions near the 5' and 3' termini of the PCR amplified full-length extrachromosomal DNA from transformant tr3. Uppercase letters are pDH33 nucleotides, lowercase letters are telomeric repeats added *in vivo*. A total of 240 bases were sequenced at the 3' terminus, 415 bases at the 5' terminus, and 1250 bases internally using primers Hyg1 and Hyg2.

Figure 5. Southern blot of *P. microspora* transformant and wild-type DNA probed with the telomeric probe TE2. Lane 33, 10 ng of pDH33. Lane Ca, 0.5 µg *Candida albicans* DNA (which has a telomeric repeat different from *P. microspora*). Lane Wt, 0.5 µg wild-type DNA. Lanes 1, 3, 4, and 5, transformant DNAs. Numbers to the right are molecular size markers.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

Definitions.

According to the National Center for Biotechnology Information (NCBI) Taxonomy Database (June 28, 1999), the fungus *Pestalotiopsis* has the following taxonomic lineage: Eukaryota; Fungi; Ascomycota; Euascomycetes; Loculoascomycetes; Dothideales; mitosporic Dothideales; Pestalotiopsis. Recent taxonomic evidence indicates that the taxonomic lineage may actually be as follows: Eukaryota; Fungi; Ascomycota; Euascomycetes; Pyrenomycetes; Xylariales; Amphisphaeriaceae; Pestalosphaeria (see Source Organism description accompanying GenBank Accession No. AF104356).

As used herein, a *Pestalotiopsis* species includes any fungus that has an 18S ribosomal RNA gene sequence with at least 80% sequence similarity to the *Pestalosphaeria* sp. NE-32 18S ribosomal RNA gene described by GenBank Accession

No. AF104356. More specifically, a *Pestalotiopsis* species includes any fungus that has an 18S ribosomal RNA gene sequence with at least 85% sequence similarity to the *Pestalosphaeria* sp. NE-32 18S ribosomal RNA gene described by GenBank Accession No. AF104356. Even more specifically, a *Pestalotiopsis* species includes any fungus that has an 18S ribosomal RNA gene sequence with at least 90% sequence similarity to the *Pestalosphaeria* sp. NE-32 18S ribosomal RNA gene described by GenBank Accession No. AF104356. Even still more specifically, a *Pestalotiopsis* species includes any fungus that has an 18S ribosomal RNA gene sequence with at least 95% sequence similarity to the *Pestalosphaeria* sp. NE-32 18S ribosomal RNA gene described by GenBank Accession No. AF104356.

As used herein, exogenous DNA refers to any DNA derived or developed outside the *Pestalotiopsis* cell undergoing transformation or derived or developed outside the *Pestalotiopsis* cell which has undergone transformation. Thus, exogenous DNA includes, but is not limited to, foreign DNA, synthetic DNA, and/or DNA from a different *Pestalotiopsis* cell than the *Pestalotiopsis* cell being transformed or which has been transformed. Foreign DNA includes, but is not limited to, any DNA from a genus other than *Pestalotiopsis* or any DNA from a species other than *P. microspora*.

As used herein, a telomerase enzyme subunit refers to any domain, or region or discrete part of a polypeptide sequence that can be equated with telomerase enzyme function.

Overview of the Invention

As set forth above, the present invention is directed to the *in vivo* addition of terminal telomeric repeats to exogenous DNA during transformation of the taxol-producing filamentous fungus *P. microspora*.

Multiple copies of the sequence 5'-TTAGGG-3', which is the telomeric repeat found in a number of filamentous fungi and in vertebrates (Henderson, 1995), are added to transforming DNA termini in a reaction that produces extrachromosomal DNAs. The DNAs do not change in size, rearrange, or undergo chromosomal integration after six months of growth with selection, but are lost after only 20 days of growth in the absence of selection. No evidence for the presence of extrachromosomal telomeric DNAs in wild-type *P. microspora* was obtained.

Transformation of *P. microspora* with an *in vivo* modified DNA amplified from one transformant by PCR is 10- to 50-fold more efficient than with the original unmodified transforming plasmid that lacks telomeric repeats. In addition to transformants harboring extrachromosomal DNAs, approximately 10% are shown to contain

5 chromosomally-integrated sequences.

The extrachromosomal DNA characterized to the fullest extent is a 5.5 kb linear molecule composed of a contiguous, apparently unmodified stretch of the transforming plasmid fused directly to terminal telomeric repeats (Figure 4b). No additional fungal sequences were detected in the extrachromosomal DNA and given the present level of

10 structural characterization (23 observed restriction fragments and approximately 1.9 kb sequenced) the presence of these unlikely.

Each *P. microspora* transformant containing extrachromosomal DNAs clearly harbors more than one type of molecule as indicated by Southern blots of transformants showing multiple bands. These are probably different conformations of the same

15 molecule, because if the transformants are ordered according to band size, approximately the same order is obtained whether the smallest or second smallest band is used. Although different covalent forms of the DNAs are possible, for example concatemers or other multimeric species, they could also be noncovalent topological forms that arise from interactions between telomeres. Indeed, the cohesive properties of oligonucleotides with

20 telomeric repeats (Williamson *et al.*, 1989), linear plasmids with telomeres (Wellinger *et al.*, 1993; Wellinger *et al.*, 1996), and telomere-capped chromosomes (Dernburg *et al.*, 1995) are well known. Particularly germane to the behavior of *P. microspora* extrachromosomal DNAs are studies by Wellinger *et al* (1993; 1996) demonstrating that linear telomeric plasmids in *Saccharomyces cerevisiae* form circular structures through

25 noncovalent interactions between telomeres with 3' overhangs. These interactions have thermal stabilities exceeding 70°C and survive typical agarose gel conditions. We believe, therefore, that circular, linear, dimeric, and higher order multimers formed by interacting telomeric termini could explain the presence of multiple extrachromosomal bands in *P. microspora* transformants.

30 The long-term stability of extrachromosomal DNAs in *P. microspora* transformants is consistent with known properties conferred on chromosomal DNA by

telomeres. These DNA-protein complexes (Fang and Cech, 1995) prevent aberrant recombination and ensure complete replication of chromosomal termini (Gall, 1995). The experiments with *P. microspora* extrachromosomal DNAs demonstrate the presence of telomeric repeats, and the structural stability of the DNAs implies the presence of fully functional telomeres. Most intriguing is the fact that exogenous DNA can apparently be placed under cellular protection and control *via* addition of partial or complete telomeres.

As discussed above, in addition to *P. microspora*, three other fungi are known to add terminal telomeric repeats to transforming DNA. These are *C. neoformans*, a basidiomycetous yeast (Edman, 1992); *H. capsulatum*, a dimorphic ascomycete (Woods and Goldman, 1992); and *F. oxysporum*, a filamentous deuteromycete (Powell and Kistler, 1990; Garcia-Pedrajas and Rocero, 1996). In the first two species and *P. microspora*, the only identified sequences donated by the host fungi are terminal telomeric repeats (Edman, 1992; Woods and Goldman, 1992). In addition to telomeric repeats, sequences homologous to an autonomously replicating sequence (*ARS*) were identified in two separate studies of *F. oxysporum*, suggesting that these could be an additional requirement for extrachromosomal replication in this fungus (Powell and Kistler, 1990; Garcia-Pedrajas and Rocero, 1996). This may explain why in *F. oxysporum*, a minority of transformants contain extrachromosomal DNAs, whereas in the other systems most transformants harbor these DNAs. Indeed, it is clear that sequence requirements for DNA replication in fungi vary (Benito *et al.*, 1995; Broach *et al.*, 1982). Perhaps *P. microspora* has relaxed sequence requirements for replication, or terminal telomeric sequences are sufficient, as is believed to be the case for *H. capsulatum* (Woods and Goldman, 1993).

High frequency *in vivo* addition of telomeric repeats to nontelomeric DNA termini is generally believed to occur only during developmentally programmed processes (Blackburn, 1995). For example, in certain ciliated protozoans, during a specific stage of development the genome undergoes massive fragmentation followed by *de novo* addition of telomeres to newly formed DNA ends (Coyne *et al.*, 1996). Outside of developmentally regulated processes, however, the *de novo* addition of telomeres to nontelomeric DNA apparently occurs rarely (Blackburn, 1995; Cooke, 1995; Melek and Shippen, 1996). Herein, we have shown that *P. microspora* possesses the biochemical mechanisms to add telomeric repeats to nontelomeric exogenous DNA and generate

extrachromosomal DNAs at a relatively high rate. It is interesting that the vast majority of fungal species, including the well-studied model organisms, have not been observed to do this (Lemke and Peng, 1995).

The following sections provide the details necessary to practice the invention as disclosed and claimed herein.

Telomeres and Telomerase

Telomeres

A large fraction of the deoxyribonucleic acid (DNA) of most higher eukaryotes is made up of repeat sequences ranging from a few copies up to millions of copies. Repeat functional sequences occur at the telomeres and centromeres of eukaryotic chromosomes.

Telomeres are specialized DNA sequences found at the ends of the chromosomes of eukaryotes which function in chromosome protection, positioning, and replication. Telomeres protect linear chromosomes from degradation and fusion to other chromosomes, and are thought to be a site of attachment to the nuclear matrix at times during the cell cycle. As chromosome caps they reduce the formation of damaged and rearranged chromosomes which arise as a consequence of recombination-mediated chromosome fusion events.

Generally, telomeres consist of tens to thousands of tandem repeats of a telomere motif sequence and associated proteins. The telomeres from all species show the same pattern: a short DNA sequence, one strand G-rich and one C-rich, that is tandemly repeated many times. The repeating telomeric unit found in *Tetrahymena* is T_2G_4 , in the ciliated protozoan *Oxytricha* it is T_4G_4 , and in yeast it is $T_{1-3}G_{1-3}$. In humans and other mammals this motif is 5'-d(TTAGGG)-3'. Sequences specific to other species such as plants may be found in Greider *et al.* (1990).

Telomeres of all human chromosomes are composed of variable length arrays of the TTAGGG repeat units with the G-rich strand oriented 5' to 3' towards the telomere. Variant telomere repeat units such as TTGGGG and TGAGGG have been identified but tend to be located at the proximal ends of human telomeres. Methods for detecting and quantitating multiple copies of a repeat sequence, such as a telomere (or centromere) repeat sequence, are provided in WO 97/14026. Methods for characterizing variability in telomere DNA by Polymerase Chain Reaction (PCR) are provided in WO 96/12821.

Telomerase

The maintenance of telomeres is required for cells to avoid replicative senescence and to continue to multiply. Chromosomes lose about 50-200 nucleotides of telomeric sequence from their ends per cell division, and the shortening of telomeres may act as a mitotic clock shortening with age both *in vitro* and *in vivo* in a replication dependent manner (Harley, 1991). Telomeric sequences can be added back to the chromosome ends, by telomere terminal transferase, also known as telomerase enzyme or simply as telomerase. Methods and compositions for increasing telomere length in normal cells to increase the proliferative capacity of cells and to delay the onset of senescence are provided in U.S. Patent Number 5,686,306.

Telomerase is a ribonucleoprotein enzyme that elongates the G-rich strand of chromosomal termini by adding telomeric repeats. This elongation occurs by reverse transcription of a part of the telomerase RNA component, which contains a sequence complementary to the telomere repeat. Following telomerase-catalyzed extension of the G-rich strand, the complementary DNA strand of the telomere is presumably replicated by more conventional means.

Telomerase is a reverse transcriptase composed of both ribonucleotide acid (RNA) and protein, wherein the RNA molecule functions as the template for the telomeric repeat. The RNA moiety of human telomerase contains the 5'-CCCTAA-3' sequence that may act as the template for *de novo* synthesis. The enzyme also contains a region that recognizes the guanine rich single strands of a DNA substrate. Methods and compositions for the determination of telomere length and telomerase activity are provided in U.S. Patent Numbers 5,489,508 and 5,707,795.

The RNA component of the telomerase enzymes of *Saccharomyces cerevisiae*, certain species of *Tetrahymena*, as well as that of other ciliates, such as *Euplotes* and *Glaucoma*, has been sequenced and reported in the scientific literature. See Singer and Gottschling, 21 Oct. 1994, Science 266:404-409; Lingner et al., 1994, Genes & Development 8:1984-1988; Greider and Blackburn, 1989, Nature 337:331-337; Romero and Blackburn, 1991, Cell 67:343-353; and Shippen-Lentz and Blackburn, 1990, Science 247:546-552; and U.S. Patent No. 5,698,686, each of which is incorporated herein by reference.

The telomerase enzymes of these ciliates synthesize telomeric repeat units distinct from that in mammals. The nucleic acids comprising the RNA of a mammalian telomerase are provided in U.S. Patent No. 5,583,016.

The functioning of telomerases seems to be activated in dividing embryonic cells and gametocytes. Telomerase activity has been identified in germ line cells and tumor cells but is repressed in differentiated somatic cells. It is now believed that the reactivation of telomerase is an essential step in tumor progression and in the immortalization of cells in culture. It is postulated that inhibition of telomerase in an immortalized cell line or in the malignant condition would cause senescence or cell death.

The introduction of synthetic oligonucleotides which mimic telomere motifs has been shown to inhibit the proliferation of immortal cells or cells that express telomerase (U.S. Patent Number 5,643,890). In fact, the single telomere motif TTAGGG exhibited greater cellular uptake and higher inhibition of proliferation than longer oligonucleotides. Methods for screening for agents which inhibit telomerase activity, including fungal telomerase activity, are provided in U.S. Patent Number 5,645,986.

Comprehensive reviews of both telomeres and telomerase are provided in U.S. Patent Numbers 5,643,890 and 5,707,795.

Telomere-Telomere Recombination

Telomere-telomere recombination provides an alternate pathway for telomere maintenance in at least some eukaryotes (Zakian, 1997). Wang *et al.* (1990) provided evidence for a telomere-telomere recombination process in yeast which involves a gene conversion event that requires little homology, occurs at or near the boundary of telomeric and non-telomeric DNA, and resembles the recombination process involved in bacteriophage T4 DNA replication.

Yeast cells which lack a functional *est1* gene exhibit a continuous decline in the terminal $(G_{1-3}T)_n$ tract, a progressive increase in the frequency of chromosome loss, and a concomitant increase in the frequency of cell death (Lundblad *et al.*, 1989). Although EST1 is not a catalytic component of telomerase (Cohn *et al.*, 1995), the same phenotypes are produced by deleting the *S. cerevisiae* telomerase RNA gene, *tlc1* (Singer and Gottschling, 1994). Although the majority of the cells in an EST1⁻ culture die, late EST1⁻ cultures give rise to derivatives that have survived the lethal consequences of the *est1*

mutation. By studying the survival of late cultures of *S. cerevisiae* cells, Lundblad *et al.* (1993) demonstrated that yeast cells have a RAD52-dependent bypass pathway by which cells can circumvent a defect in the EST1-mediated pathway for yeast telomere replication. Most of the surviving cells have very short telomeres but acquire long tandem arrays of subtelomeric repeats by gene conversion. The researchers concluded that “even when the primary pathway for telomere replication is defective, an alternative backup pathway exists that restores sufficient telomere function for continued cell viability.”

Although deletion of the telomerase RNA gene, *ter1*, in the yeast *Kluyveromyces lactis* also results in the gradual loss of telomeric repeats and progressively declining cell growth capability, some cells are able to continue growing without telomerase. McEachern *et al.* (1996) proposed that shortened, terminal telomeric repeat tracts become uncapped, promoting recombinational repair between them to regenerate lengthened telomeres in survivors. They termed this process telomere cap-prevented recombination (CPR).

Artificial Chromosomes

Components of Artificial Chromosomes

Artificial chromosomes are man-made linear DNA molecules constructed from essential DNA sequence elements that are responsible for the proper replication and partitioning of natural chromosomes (Murray *et al.*, 1983). The essential elements necessary to construct artificial chromosomes include:

- 1) a centromere, which is the site of kinetochore assembly and is responsible for the proper distribution of replicated chromosomes at cell division (*i.e.*, mitosis and meiosis);
- 2) two telomeres, the structures at the ends of a chromosome, which are needed to prevent the chromosome from being nibbled away by exonucleases;
- 3) an origin of replication, also known as Autonomous Replication Sequences (ARS), which are the positions along the chromosome at which DNA replication initiates.

The construction of functional artificial chromosomes provides an alternate method for transforming cells. Artificial chromosome vectors can be constructed to include gene sequences capable of producing specific polypeptides, wherein the gene

sequences can include extremely long stretches of exogenous DNA. Of course, selectable marker genes can also be included in such artificial chromosomes to aid in the selection of transformed cells.

Use of artificial chromosome recombinant molecules as vectors solves many of the problems associated with alternative transformation technologies which are used to introduce new DNA into higher eukaryotic cells. Since artificial chromosomes are maintained in the cell nucleus as independently replicating DNA molecules, sequences introduced on such vectors are not subject to the variable expression due to integration position effects. In addition, the delivery of artificial chromosomes to the nucleus of a cell as intact, unbroken, double-stranded DNA molecules with telomeric ends ensures that the introduced DNA can be maintained stably in that form and that rearrangements should not occur. Furthermore, artificial chromosome vectors will be stably maintained in the nucleus through meiosis and will be available to participate in homology-dependent meiotic recombination. Exogenous DNA introduced via artificial chromosome vectors can be delivered to practically any cell without host range limitations, in contrast to some other transformation methods such as the *Agrobacterium*-mediated DNA transfer systems.

Yeast Artificial Chromosomes

Yeast artificial chromosomes (YACs) are genetically engineered chromosomes that contain the essential DNA sequence elements of *Saccharomyces* and segments of exogenous DNAs that are much larger than those accepted by conventional cloning vectors.

YACs are generated from synthetic minichromosomes that contain a yeast centromere, a replication origin, and fused telomeres. The circular chromosome also contains three marker genes (*m1*, *m2*, and *m3*), which when expressed, allow selection of the cells carrying the plasmid and two specific sites (Burke et al, 1987). These two sites allow specific restriction endonucleases to break the molecule. Cleavage at one site opens the ring, while cleavage at the second site generates centric and acentric fragments with ends that will accept exogenous DNA fragments. Once these ends are ligated, an artificial chromosome is generated with a short and a long arm, with the long arm containing the spliced segment of exogenous DNA to be cloned. Such artificial chromosomes are

distributed normally during subsequent yeast divisions creating colonies containing the YACs. In cells possessing the insert, the *m1* and *m3* markers are expressed, but the damaged M2 is not, allowing religated YACs to be distinguished from unbroken plasmids. For further descriptions of this process, see T. A. Brown, Gene Cloning, Second Edition, Chapman & Hall (1990), U.S. Patent Number 4,889,806 and U.S. Patent Number 5,270,201.

Telomeric fragments of human DNA, including the sequence for the human telomere, ranging in size from 50 to 250 kilobases have been cloned into *Saccharomyces cerevisiae* using YAC vectors (see, e.g., Riethman *et al.*, 1989; Guerrini *et al.*, 1990).

YAC vectors can be constructed according to the methods detailed in U.S. Patent Nos. 4,889,806 and 5,270,201.

Yeast ARSs have not been found to replicate in filamentous fungi (Fincham, 1989).

Mammalian Artificial Chromosomes

The controlled construction of mammalian artificial chromosomes (MACs) has been difficult because, with the exception of telomeres, the corresponding essential elements in mammals have not been fully defined. Higher eukaryotes (e.g., mammals), in contrast to yeast, contain repetitive DNA sequences which form a boundary at both sides of the centromere. This highly repetitive DNA interacting with certain proteins, especially in animal chromosomes, creates a genetically inactive zone (heterochromatin) around the centromere. This pericentric heterochromatin keeps any selectable marker gene at a considerable distance, and thus repetitive DNA prevents the isolation of centromeric sequences by chromosome "walking." Alpha-satellite (alphoid) DNA forms a family of repeated DNA sequences found in amounts varying from 500 kb to 5 mb at the centromeres of human chromosomes. Alphoid sequences consist of a repeated 171 bp monomer that exhibits chromosome-specific variation in nucleotide sequence and higher order repeat arrangement.

U.S. Patent Number 5,288,625 reports that a cell line which contains a dicentric chromosome, one of the centromeres of which contains a segment of human DNA, can be treated so as to isolate the centromere which contains the human DNA on a chromosome away from other mammalian chromosomes. Using a mouse lung fibroblast cell which

contains such a dicentric chromosome wherein the centromere is linked to a dominant selectable marker (*e.g.*, aminoglycoside-3' phosphotransferase-II), the inventor was able to isolate derivative cell lines which stably replicated a chromosome containing only centromeres comprising cloned human DNA.

5 Harrington *et al.* (1997) have constructed stable human artificial chromosomes by cotransfecting large synthetic arrays of alphoid repeats, telomere repeats, and random genomic DNA fragments into human cultured cells. In general, the resultant minichromosomes acquired host sequences by means of either a chromosome truncation event or rescue of an acentric fragment, but in one case minichromosome formation was
10 by a *de novo* mechanism. The inclusion of uncharacterized genomic DNA in the transfection mixture raises the possibility that sequences other than the transfected alphoid and telomere DNA contributed to chromosome formation.

To construct YAC-based mammalian artificial chromosomes, Ikeno *et al.* (1998) introduced telomere repeats and selectable markers into a 100 kb YAC containing human
15 centromeric DNA. The resultant YAC, which has regular repeat sequences of alpha-satellite DNA and centromere protein B (CENP-B) boxes, efficiently formed MACs that segregated accurately and bound CENP-B, CENP-C, and CENP-E. The MACs appear to be about 1-5 Mb in size and contain YAC multimers. It is not known whether the MACs are linear or circular. The data from structural analyses of the MACs by FISH and
20 Southern blot hybridization suggest that the introduced YAC DNA itself must have been multimerized by recombination and/or amplification.

Genetic Transformation Methods

Production of Transgenic Animals

Transgenic animals are genetically modified animals into which cloned genetic
25 material has been transferred. The cloned genetic material is often referred to as a transgene. The transgene may consist of nucleic acid sequences derived from the genome of the same species or of a different species, including non-animal species, than the species of the target animal.

The development of transgenic technology allows investigators to create
30 mammals of virtually any genotype and to assess the consequences of introducing specific exogenous nucleic acid sequences on the physiological and morphological characteristics

of the transformed animals. The availability of transgenic animals permits cellular processes to be influenced and examined in a systematic and specific manner not achievable with most other test systems. For example, the development of transgenic animals provides biological and medical scientists with models that are useful in the study of disease. Such animals are also useful for the testing and development of new pharmaceutically active substances. Gene therapy can be used to ameliorate or cure the symptoms of genetically-based diseases.

Transgenic animals can be produced by a variety of different methods including transfection, electroporation, microinjection, biolistics (also called gene particle acceleration or microprojectile bombardment), gene targeting in embryonic stem cells and recombinant viral and retro viral infection (*see, e.g.,* U.S. Patent No. 4,736,866; U.S. Patent No. 5,602,307; Mullins et al., *Hypertension* 22(4):630-633 (1993); Brenin et al., *Surg. Oncol.* 6(2)99-110 (1997); Tuan (ed.), *Recombinant Gene Expression Protocols*, Methods in Molecular Biology No. 62, Humana Press (1997)).

The term "knock-out" generally refers to mutant organisms which contain a null allele of a specific gene. The term "knock-in" generally refers to mutant organisms into which a gene has been inserted through homologous recombination. The knock-in gene may be a mutant form of a gene which replaces the endogenous, wild-type gene.

A number of recombinant rodents have been produced, including those which express an activated oncogene sequence (U.S. Patent No. 4,736,866); express simian SV 40 T-antigen (U.S. Patent No. 5,728,915); lack the expression of interferon regulatory factor 1 (IRF-1) (U.S. Patent No. 5,731,490); exhibit dopaminergic dysfunction (U.S. Patent No. 5,723,719); express at least one human gene which participates in blood pressure control (U.S. Patent No. 5,731,489); display greater similarity to the conditions existing in naturally occurring Alzheimer's disease (U.S. Patent No. 5,720,936); have a reduced capacity to mediate cellular adhesion (U.S. Patent No. 5,602,307); possess an bovine growth hormone gene (Clutter et al., *Genetics* 143(4):1753-1760 (1996)); and are capable of generating a fully human antibody response (McCarthy, *The Lancet* 349(9049):405 (1997)).

While rodents, especially mice and rats, remain the animals of choice for most transgenic experimentation, in some instances it is preferable or even necessary to use

alternative animal species. Transgenic procedures have been successfully utilized in a variety of non-murine animals, including sheep, goats, pigs, dogs, cats, monkeys, chimpanzees, hamsters, rabbits, cows and guinea pigs (*see, e.g., Kim et al., Mol. Reprod. Dev.* 46(4):515-526 (1997); Houdebine, *Reprod. Nutr. Dev.* 35(6):609-617 (1995);
5 Petters, *Reprod. Fertil. Dev.* 6(5):643-645 (1994); Schnieke et al., *Science* 278(5346):2130-2133 (1997); and Amoah, *J. Animal Science* 75(2):578-585 (1997)).

The method of introduction of nucleic acid fragments into recombination competent mammalian cells can be by any method which favors co-transformation of multiple nucleic acid molecules. Detailed procedures for producing transgenic animals
10 are readily available to one skilled in the art, including the recitations in U.S. Patent No. 5,489,743 and U.S. Patent No. 5,602,307.

2. Production of Transgenic Plants

Transgenic plants can be produced by a variety of different transformation methods including, but not limited to, electroporation; microinjection; microprojectile
15 bombardment, also known as particle acceleration or biolistic bombardment; viral-mediated transformation; and Agrobacterium-mediated transformation (*see, e.g., U.S. Patent Numbers 5,405,765, 5,472,869, 5,538,877, 5,538,880, 5,550,318, 5,641,664, 5,736,369 and 5,736,369; Watson et al., Recombinant DNA, Scientific American Books* (1992); Hinchey et al., *Bio/Tech.* 6:915-922 (1988); McCabe et al., *Bio/Tech.* 6:923-926
20 (1988); Toriyama et al., *Bio/Tech.* 6:1072-1074 (1988); Fromm et al., *Bio/Tech.* 8:833-839 (1990); Mullins et al., *Bio/Tech.* 8:833-839 (1990); and Raineri et al., *Bio/Tech.* 8:33-38 (1990)).

Genes successfully introduced into plants using recombinant DNA methodologies include, but are not limited to, those coding for the following traits: seed storage proteins,
25 including modified 7S legume seed storage proteins (U.S. Patent Numbers 5,508,468, 5,559,223 and 5,576,203); herbicide tolerance or resistance (U.S. Patent Numbers 5,498,544 and 5,554,798; Powell et al., *Science* 232:738-743 (1986); Kaniewski et al., *Bio/Tech.* 8:750-754 (1990); Day et al., *Proc. Natl. Acad. Sci. USA* 88:6721-6725 (1991)); phytase (U.S. Patent Number 5,593,963); resistance to bacterial, fungal,
30 nematode and insect pests, including resistance to the lepidoptera insects conferred by the Bt gene (U.S. Patent Numbers 5,597,945 and 5,597,946; Hilder et al., *Nature* 330:160-

163; Johnson et al., *Proc. Natl. Acad. Sci. USA*, 86:9871-9875 (1989); Perlak et al., *Bio/Tech.* 8:939-943 (1990)); lectins (U.S. Patent Number 5,276,269); and flower color (Meyer et al., *Nature* 330:677-678 (1987); Napoli et al., *Plant Cell* 2:279-289 (1990); van der Krol et al., *Plant Cell* 2:291-299 (1990)).

5 3. Homologous Recombination

Genes can be introduced in a site directed fashion using homologous recombination. This can be used in the creation of a transgenic animal, wherein the animal would be mutated, and the phenotype of the mutation could be studied for purposes of drug screening, investigating physiologic processes, developing new products and the like. Papers discussing homologous recombination are discussed in U.S. Patent No. 5,413,923.

Homologous recombination permits site-specific modifications in endogenous genes and thus inherited or acquired mutations may be corrected, and/or novel alterations may be engineered into the genome. The application of homologous recombination to gene therapy depends on the ability to carry out homologous recombination or gene targeting in normal, somatic cells for transplantation.

To prepare cells for homologous recombination, embryonic stem cells or a stem cell line may be obtained. Cells other than embryonic stem cells can be utilized (*e.g.* hematopoietic stem cells etc.) (See U.S. Patent No. 5,589,369 for more examples). The cells may be grown on an appropriate fibroblast fetal layer or grown in the presence of leukemia inhibiting factor (LIF) and then used. The embryonic stem cells may be injected into a blastocyst, that has been previously obtained, to provide a chimeric animal. The main advantage of the embryonic stem cell technique is that the cells transfected with the "transgene" can be tested prior to reimplantation into a female animal for gestation for integration and the effect of the transgenes. By subsequent cross-breeding experiments, animals can be bred which carry the transgene on both chromosomes. If mutations are incorporated into the transgenes which block expression of the normal gene production, the endogenous genes can be eliminated by this technique and functional studies can thus be performed.

30 Methods for intracellularly producing DNA segments by homologous recombination of smaller overlapping DNA fragments and transgenic mammalian cells

and whole animals produced by such methods are disclosed in U.S. Patent No. 5,612,205. Cell lines useful for analysis of human homologous interchromosomal recombination are provided in U.S. Patent Application No. 5,554,529.

Homologous recombination can also proceed extrachromasomally, which may be of benefit when handling large gene sequences (*e.g.*, larger than 50 kb). Methods of performing extrachromosomal homologous recombination are described in U.S. Patent No. 5,721,367.

Homologous recombination and site-directed integration in plants are discussed in U.S. Patent Nos. 5,451,513, 5,501,967 and 5,527,695.

Determining Sequence Similarity

Nucleic acid molecules of the invention include the nucleotide sequences coding for a *Pestalotiopsis* telomerase enzyme or a subunit of a *Pestalotiopsis* telomerase enzyme. Any nucleic acid sequence which specifically hybridizes to such nucleic acid molecules such that the sequence remains stably bound to said nucleic acid molecules under highly stringent or moderately stringent conditions is also encompassed within this invention. Stringent and moderately stringent conditions are those commonly defined and available, such as those defined by Sambrook *et al.* (1989) or Ausubel *et al.* (1995). The precise level of stringency is not important, rather, conditions should be selected that provide a clear, detectable signal when specific hybridization has occurred.

Hybridization is a function of sequence identity (homology), G+C content of the sequence, buffer salt content, sequence length and duplex melt temperature ($T[m]$) among other variables. See, Maniatis *et al.* (1982). With similar sequence lengths, the buffer salt concentration and temperature provide useful variables for assessing sequence identity (homology) by hybridization techniques. For example, where there is at least 90 percent homology, hybridization is commonly carried out at 68° C in a buffer salt such as 6XSSC diluted from 20XSSC. See Sambrook *et al.* (1989). The buffer salt utilized for final Southern blot washes can be used at a low concentration, *e.g.*, 0.1XSSC and at a relatively high temperature, *e.g.*, 68° C, and two sequences will form a hybrid duplex (hybridize). Use of the above hybridization and washing conditions together are defined as conditions of high stringency or highly stringent conditions. Moderately stringent conditions can be utilized for hybridization where two sequences share at least about 80 percent homology.

In particular, specific hybridization occurs under conditions in which a high degree of complementarity exists between a nucleic acid comprising the sequence of an isolated sequence and another nucleic acid. With specific hybridization, complementarity will generally be at least about 70%, 75%, 80%, 85%, preferably about 90-100%, or most preferably about 95-100%.

As used herein, homology or identity is determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn and tblastx (Karlin *et al.* Proc. Natl. Acad. Sci. USA 87: 2264-2268 (1990) and Altschul, S. F. J. Mol. Evol. 36: 290-300(1993), both of which are herein incorporated by reference) which are tailored for sequence similarity searching. The approach used by the BLAST program is to first consider similar segments between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (Nature Genetics 6: 119-129 (1994)) which is herein incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (i.e., the statistical significance threshold for reporting matches against database sequences), cutoff, matrix and filter are at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff *et al.* Proc. Natl. Acad. Sci. USA 89: 10915-10919 (1992), herein incorporated by reference). For blastn, the scoring matrix is set by the ratios of M (i.e., the reward score for a pair of matching residues) to N (i.e., the penalty score for mismatching residues), wherein the default values for M and N are 5 and -4, respectively.

The nucleic acids of the present invention can be used in a variety of ways in accordance with the present invention. For example, they can be used as nucleic acid probes to screen other cDNA and genomic DNA libraries so as to select by hybridization other DNA sequences that encode homologous sequences. Contemplated nucleic acid probes could be RNA or DNA labeled with radioactive nucleotides or by non-radioactive methods (for example, biotin). Screening may be done at various stringencies (through manipulation of the hybridization T_m , usually using a combination of ionic strength, temperature and/or presence of formamide) to isolate close or distantly related homologs.

The nucleic acids may also be used to generate primers to amplify cDNA or genomic DNA using polymerase chain reaction (PCR) techniques. The nucleic acid sequences of the present invention can also be used to identify adjacent sequences in the genome, for example, flanking sequences and regulatory elements. The nucleic acids may also be used to generate antisense primers or constructs that could be used to modulate the level of gene expression. The amino acid sequence may be used to design and produce specific antibodies.

Taxol Biosynthesis

The first dedicated step in the biosynthesis of taxol in *T. brevifolia* is the cyclization of the universal diterpene precursor geranylgeranyl diphosphate to taxa-4(5),11(12)-diene (Koepp *et al.*, 1995). The operationally soluble cyclization enzyme taxa-4(5),11(12)-diene synthase, also called taxadiene synthase, isolated from the Pacific yew tree has been purified and characterized (Hezari *et al.*, 1995). Non-denaturing, followed by denaturing, polyacrylamide gel electrophoresis, in combination with gel permeation chromatography, allowed identification of taxadiene synthase as a monomeric protein of molecular weight 79,000.

A cDNA (pTb42.1) isolated from *T. brevifolia* and functionally expressed in *Escherichia coli* yielded a protein that was confirmed to be taxadiene synthase (Wildung and Croteau, 1996). The sequence specifies an open reading frame of 2586 nucleotides and the complete deduced polypeptide, including a long presumptive plastidial targeting peptide, contains 862 amino acid residues and has a molecular weight of 98,303. Sequence comparisons with monoterpene, sesquiterpene, and diterpene cyclases of plant origin indicate a significant degree of similarity between these enzymes. The taxadiene synthase most closely resembles (46% identity, 67% similarity) abietadiene synthase, a diterpene cyclase from grand fir.

Huang *et al.* (1998) report a method for the heterologous overexpression of cDNA encoding taxadiene synthase in *Escherichia coli* using a thioredoxin fusion expression system, which increases the solubility of expressed protein. The maximal production attained was 13 mg of purified, active fusion protein per 500 ml culture of *E. coli* BL21(DE3)/pET32TX.

The cyclization step discussed above is followed by extensive, largely oxidative,

modification of this diterpene olefin to taxa-4(20), 11(12)-dien-5 alpha-ol. The structure of the first oxygenated intermediate on the taxol pathway establishes that the hydroxylation reaction proceeds with an unusual double bond migration that limits the mechanistic possibilities for subsequent elaboration of the oxetane moiety of taxol (Hefner *et al.*, 1996). The reaction is catalyzed by a cytochrome P450, suggesting that the seven remaining oxygenation steps in taxol biosynthesis may involve similar catalysts. Hefner *et al.* (1996) suggest that because the first oxygenation step is slow relative to subsequent metabolic transformations, that it may be possible to speed taxol biosynthesis by isolating and manipulating the gene for the taxadiene-5-hydroxylase that catalyzes this reaction.

The third step of taxol biosynthesis has been identified as the acetyl CoA-dependent conversion of the alcohol to the corresponding acetate ester (Hezari and Croteau, 1997). Zocher *et al.* (1996) reported the enzymatic acetylation of 10-deacetylbaccatin-III to baccatin-III in crude extracts from root of *Taxus baccata*.

In a review of the first three steps of taxol biosynthesis, including the responsible enzymes, Hezari and Croteau (1997) suggest that the complete pathway can be defined by a systematic, stepwise approach at the cell-free enzyme level. When combined with *in vivo* studies to determine contribution to pathway flux, the authors further suggest that the flow steps can be targeted for gene isolation and subsequent overexpression in *Taxus* to improve the yield of taxol and related compounds.

The Production of Taxanes

As discussed above in detail, taxol is naturally produced by yew plant species and by microorganisms such *P. microspora*. In an effort to produce greater quantities of taxanes, such as taxol, a number of different artificial systems have been utilized.

U.S. Patent No. 5,322,779 provides *in vitro* methods of producing taxol using the fungus *Taxomyces andreanae*, wherein the fungus is isolated from tissue fragments from a tree of the genus *Taxus*. U.S. Patent No. 5,310,672 provides methods for the production of taxol and taxol precursors from *in vitro* propagated cultures of somatic embryos derived from explants of the genus *Taxus*, especially from *T. brevifolia*.

Taxus suspension cultures have been used for the production of taxanes such as taxol (*see, e.g.*, Hezari *et al.*, 1997; U.S. Patent No. 5,665,576). Using suspension cultures of *Taxus cuspidata* grown in either shake flasks or Wilson-type reactors in which

bubbled air provided both agitation and mixing, Pestchanker *et al.* (1996) obtained taxol titers of 22 mg per liter in 20 days.

U.S. Patent No. 5,279,953 provides a method for improved *in vivo* production of taxanes by inoculation of tissue of the genus *Taxus* with virulent or avirulent strains of *Agrobacterium*. Incomplete harvesting of galls permits regeneration on the trees, resulting in a renewable resource. The net yield increase demonstrated was 2 to 6 times normal (4 to 6 times for the galls themselves), did not require special growth hormones or media, did not result in sacrificing the tree, and can be started in the sapling stage.

EXAMPLES

Example 1. Source of *Pestalotiopsis microspora*.

P. microspora was isolated from the inner bark of a Himalayan yew tree (*Taxus wallachiana*) near Kathmandu, Nepal as previously described (Strobel *et al.*, 1996). Fungal material for all experiments was derived from a single germinating conidium from an isolate designated NE-32.

Example 2. Transforming DNA.

Plasmid pDH33 is a derivative of pBR322 containing the *Aspergillus niger glaA* promoter and *Aspergillus nidulans trpC* terminator fused to the *E. coli* hygromycin resistance gene (Smith *et al.*, 1990). Plasmid pDH25 is a derivative of pBR322, but has the *A. nidulans trpC* promoter fused to hygromycin resistance gene (Cullen *et al.*, 1987).

Oligonucleotides were synthesized by Operon Technologies (Alameda, CA) or by the Iowa State University DNA Sequencing Facility. DNA sequencing was performed by the Iowa State University DNA Sequencing Facility with an ABI 377 DNA Sequencer (Perkin Elmer).

Example 3. Protoplasting and Transformation.

Materials and Methods.

Protoplasts of *P. microspora* were produced by cell wall digestion of two day old mycelium (grown in potato dextrose broth, PDB) in digest buffer (20 mM K₂HPO₄, 50 mM MgSO₄•7H₂O, 0.7 M sorbitol, pH 6.3) containing 20 mg/ml Novozyme 234

(Calbiochem) and 20 mg/ml β -glucuronidase (Sigma) at 30°C for 90 min with 130 rpm shaking, in a proportion of 2 ml per 100 mg mycelium wet weight. Protoplasts were filtered through glass wool, collected by centrifugation (825g, 7 min, 20°C), washed in digest buffer, resuspended in 0.5 ml transformation buffer (10 mM Tris HCl, 0.1 M CaCl₂, 0.7 M sorbitol, pH 7.5), and counted with a hemocytometer.

To transform, 3×10^6 protoplasts suspended in 0.1 ml transformation buffer were incubated for 15 min at room temperature with 0.5 μ g pDH33, and for an additional 20 min at room temperature after addition of 0.4 ml 40% PEG 3350 (W/V in transformation buffer) in small increments with no mixing (important). Transformed protoplasts were incubated for 4 h at room temperature in 0.4 ml PDB containing 0.6 M sucrose and plated onto potato dextrose agar (PDA) containing 0.6 M sucrose and 200 μ g/ml hygromycin. Germination of untransformed protoplasts was inhibited by 50 μ g/ml hygromycin. After 7 days, transformants were transferred to PDA containing 200 μ g/ml hygromycin.

Results.

Treatment of *P. microspora* protoplasts with CaCl₂ and PEG in the presence of plasmid pDH33 typically yielded 50 to 1000 hygromycin-resistant transformants per microgram of pDH33 per 4×10^5 regenerating protoplasts. At least 10-fold more transient transformants, which clearly demonstrated resistance but for only one to four days, were also observed. Comparable transformation efficiencies were obtained with pDH25, a similar plasmid but with a different promoter controlling the hygromycin resistance gene. In control transformations with transforming DNA omitted, no resistant *P. microspora* colonies were ever observed, indicating that spontaneous resistance to hygromycin arises at a frequency of less than one in 4×10^5 .

Example 4. Phenotypic Characterization of Transformants.

Materials and Methods.

Stability of the transformant phenotype during vegetative growth in the absence of selection was measured in a two step procedure. First, four 3 mm x 3 mm blocks from different regions of the growing margin of *P. microspora* transformant colonies were transferred to PDA (lacking hygromycin) every 5 days for 35 days, with incubation at 24°C. Second, at the time of each transfer, retention of the transformant phenotype (hygromycin resistance) was assessed by transferring four blocks of mycelium from the

growing margin to PDA containing 0, 100, 200, 400, 600, 800, and 1000 µg/ml hygromycin and calculating colony areas after 7 days of growth at 24°C.

Conidiation was induced by placing blocks of mycelia from *P. microspora* transformants on carnation leaf agar (1.5% water agar, W/V) containing γ-irradiated carnation leaf pieces (Nelson *et al.*, 1983). After 10-14 days of growth at room temperature, conidia were collected from acervuli and suspended in 0.1% Tween 20 (which prevents conidia from adhering to pipette tips and Eppendorff tubes), and counted with a hemocytometer. The fraction of conidia resistant to hygromycin was measured by comparing the germination rate on PDA with that on PDA containing 200µg/ml hygromycin.

To measure the stability of the resistant phenotype in the presence of selection, two 3 mm x 3 mm blocks of growing edge mycelia from each *P. microspora* transformant were transferred to fresh PDA containing 200µg/ml hygromycin every 7 days for 6 months, with all incubations at 24°C. At the time of each transfer, donor colony areas were calculated as a measure of resistance.

Results.

1. Southern Blot Analyses of Transformants.

Wild-type *P. microspora* and pDH33 transformants were examined by Southern blotting using radio labeled pDH33 as probe. No hybridization signal was visible from wild-type *P. microspora* DNA (Figure 1, lane Wt). Unexpectedly, extrachromosomal DNAs having lower molecular weights than fragmented chromosomal DNA were present in 90% of transformants (Figure 1, lanes 1-8). Each of these exhibited three to six extrachromosomal DNAs of discrete sizes ranging from approximately six to 30 kb. Extrachromosomal DNA sizes usually differed from one transformant to another. The quantity of extrachromosomal DNA varied from band to band within individual transformants and from transformant to transformant. In no transformant was the smallest extrachromosomal DNA the most abundant.

When transformants were ordered according to band size, approximately the same order was obtained whether the smallest or second smallest band was used, suggesting that extrachromosomal DNAs within individual transformants may be different conformations of the same molecule. The extrachromosomal DNAs nearly always differed in size from

circular and linear pDH33 (Figure 1, lane 33), ruling out contamination by pDH33 and indicating that *P. microspora* was not replicating unmodified pDH33. Because DNA samples were subjected to heavy RNase treatment, hybridization signal could not be attributed to the presence of contaminating RNA. Several transformants containing
5 extrachromosomal DNAs also exhibited hybridization signal in the region of genomic DNA (Figure 1, lanes 2,3,7, and 8), and two transformants exhibited hybridization signal only in the region of genomic DNA (Figure 3a, lane 1; data not shown for tr19).

2. Phenotypic Characterization of Transformants.

Phenotypic characterization was undertaken to determine whether the stability of
10 resistance in transformants was consistent with Southern blot evidence for extrachromosomal DNAs.

After growth without selection for 35 days, two transformants (tr14 and tr19) exhibiting hybridization signal only in the region of genomic DNA retained resistance (Figure 2a). When combined with Southern blots of restricted DNA demonstrating
15 chromosomal integration of transforming DNA (data not shown), this indicated that these two transformants were integrants. In contrast, after 20 days of growth without selection, transformants with extrachromosomal bands lost all observable resistance (Figure 2a). Hybridization signal comigrating with genomic DNA in this latter type of transformant was therefore presumed to be due to the presence of large extrachromosomal DNAs
20 unresolvable from fragmented chromosomal DNA under the electrophoresis conditions utilized. Consistent with these phenotypic observations, Southern blots of DNA from transformants after 35 days of growth without selection revealed retention of hybridizing signal in an integrant (Figure 3a, lane 2) but complete loss of signal from transformants with extrachromosomal DNAs (Figure 3a, lanes 5, 8, and 11).

25 Three additional phenotypic characteristics distinguished integrants from transformants containing extrachromosomal DNAs. Integrants demonstrated higher resistance to hygromycin (Figure 2b), produced more abundant aerial hyphae, and when grown without selection, produced a much larger proportion of resistant conidia (1 in 2 vs. 1 in 10^2 - 10^5 for transformants with extrachromosomal DNAs).

30 When germinated on non-selective media, and subsequently transferred to selective media, 100% of the conidia sampled (*i.e.*, 74) from resistant single-spore

colonies of two different integrants were resistant. Retention of resistance after growth without selection is characteristic of chromosomally integrated selective markers, while loss of resistance under these conditions is typical of selective markers on extrachromosomal elements (Lemke and Peng, 1995). We conclude, from both Southern analyses and phenotypic data, that the most common fate of plasmid pDH33 in *P. microspora* transformants is extrachromosomal replication after *in vivo* modification, with a less frequent outcome being chromosomal integration.

3. Stability of Extrachromosomal DNAs in the Presence of Selection.

Transformants containing extrachromosomal DNAs were replated on selective media weekly for six months. During this time, there was no observable change in colony growth rates (data not shown). Southern analyses of three transformants, before and after the six month period, revealed no observable change in size or amounts of the DNAs and no evidence of chromosomal integration (Figure 3a, lanes 3, 6, and 9 before the six month period; lanes 4, 7, and 10 after the six month period).

Southern analysis of restricted DNA from the three transformants before and after the six month period identified 26 restriction fragments that showed no visible change in size and provided no evidence for rearrangements of extrachromosomal DNAs (Figure 3b for transformant tr10; data not shown for transformants tr13 and tr 15). Taken together, these data suggest that extrachromosomal DNAs in *P. microspora* transformants retain the ability to replicate and demonstrate a high degree of structural stability during long-term vegetative growth in the presence of selection.

Example 5. Preparation and Analysis of *P. microspora* DNA

Materials and Methods.

DNA extraction from lyophilized mycelium was accomplished as described by Raeder and Broda (1985). Mycelium for DNA extraction was grown in modified Czapek's medium (2 g NaNO₃, 1 g KH₂PO₄, 500 mg MgSO₄•7H₂O, 500 mg KCl, 10 mg FeSO₄•7H₂O, 500 mg yeast extract, and 100 mg sucrose per liter H₂O with addition of 20 ¹³ g/ml hygromycin for transformants) for 4 days without shaking, collected by centrifugation (10,500 g, 20 min), and lyophilized for 2 days. Restriction digests (*Bam*HI, *Ban*I, *Cl*aI, *Eco*RI, *Sac*I, *Sma*I, *Xba*I, and *Xho*I; Promega) were carried out according to instructions from the supplier. For Southern blots, ³²P-labeled probe was synthesized by

the random primed reaction using *Xba*I-linearized pDH33 as template and the Klenow fragment according to instructions from the supplier (Boehringer Mannheim).

Fractionated DNA in agarose gels was depurinated, denatured, neutralized, transferred overnight in 20X SSC (1X SSC is 0.015 M sodium citrate, 0.15 M sodium chloride, pH 7) to Hybond-N nylon membrane (Amersham), and fixed by baking for 2 h at 80°C, all according to standard procedures (Sambrook *et al.*, 1989).

Membranes were prehybridized for 1 h at 68°C in a solution consisting of 6X SSC, 5X Denhardt's reagent (1X Denhardt's reagent is 200 mg Ficoll, 200 mg polyvinylpyrrolidone, and 200 mg bovine serum albumin per liter water), 0.5% SDS (W/V), 8% PEG 6000 (W/V), 100 µg/ml sheared denatured salmon sperm DNA (Sigma). After prehybridization labeled probe was added to a final concentration of 20 ng/ml and incubation was continued at 68°C for approximately 18 h. Hybridized membranes were washed twice at room temperature for 15 min in 100 ml 2X SSC/0.1% SDS, washed once at 68°C in 100 ml 0.1X SSC/0.1% SDS, and exposed to Hyperfilm-MP (Amersham) with an intensifying screen at -70°C. Southern blots to detect telomeric sequences employed an 18 base synthetic oligonucleotide probe (TE2, 5'-(CCCTAA)₃-3') 5' end labeled with ³²P using T4 polynucleotide kinase according to procedures recommended by the supplier (Promega).

DNA fractionated on agarose gels was depurinated and transferred in alkaline solution overnight to Hybond-N+ nylon membrane (Amersham). Membranes were prehybridized for 30 min at 44°C in the hybridization solution described above but lacking salmon sperm DNA. ³²P-labeled probe was added to a final concentration of 0.25 ng/ml and incubation was continued for approximately 18 h at 44°C. Hybridized membranes were washed twice for 15 min at room temperature in 100 ml 2X SSC/0.1% SDS and exposed to Hyperfilm-MP (Amersham) with an intensifying screen at -70°C or to a phosphor screen (Molecular Dynamics).

Example 6. Amplification of *P. microspora* DNA in *E. coli*.

Materials and Methods.

Enzymatic circularization of *P. microspora* transformant DNA was accomplished essentially as described by Powell and Kistler (1990) except using 0.3 to 1.5 µg transformant total DNA per reaction. *P. microspora* transformant total DNA (untreated or

circularized) was transformed into SURE strain *E. coli* cells (Stratagene) by electroporation using an Electro Cell Manipulator 600 electroporator (BTX) according to suggestions from the manufacturer, plated on LB agar (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, and 100 µl 10 N NaOH per liter) containing 50 µg/ml ampicillin, and incubated overnight at 37°C. Plasmids from *E. coli* were prepared by standard alkaline lysis (Sambrook *et al.*, 1989) or with a Wizard Plus SV Miniprep kit (Promega).

Example 7. Polymerase Chain Reaction

Materials and Methods.

PCR amplifications were carried out in a Model 480 DNA Thermal Cycler (Perkin Elmer) in 50 µl reactions containing 2 mM Mg²⁺ and approximately 100 ng of total transformant DNA.

Amplifications with primer TE2 (final concentration 0.8 µM, sequence described above) utilized 2 µl of ELONGASE Enzyme Mix (Gibco/BRL Life Technologies) and employed temperature cycling parameters of 94°C for 30 sec, followed by 30 cycles of 94°C for 30 sec, 46°C for 30 sec, and 68°C for 10 min.

Amplifications containing primers TE2 and either Hyg1 (5'-TTACCCGCAGGACATATCCACG-3') (SEQ ID. NO: 1) or Hyg2

(5'-ATAGCTGCGCCGATGGTTTCTA-3') (SEQ ID. NO: 2) employed 0.2 µM final concentrations of each primer, 1 µl of enzyme mix, and a cycling protocol of 94°C for 30 sec followed by 25 cycles of 94°C for 30 sec, 50°C for 30 sec, and 68°C for 10 min.

PCR products were purified using a QIAquick PCR purification kit (Qiagen).

Results.

1. PCR Amplification of Extrachromosomal DNA.

To avoid potential ambiguities associated with inferring the structure of extrachromosomal DNAs from enzymatically circularized DNA replicated in *E. coli*, we used methods based on PCR.

Beginning with the assumption that linear extrachromosomal DNAs contain telomeric repeats at each terminus, a primer (TE2) complementary to the 5'-TTAGGG-3' telomeric sequence was used in PCR amplifications of *P. microspora* transformant total DNA. Although this primer should also anneal to chromosomal telomeres, only

extrachromosomal DNAs were expected to amplify because chromosomal DNA fragments have at most a single telomeric terminus (genomic DNA samples are typically degraded to about 50 kb). To minimize the technical difficulties of long PCR total DNA from transformant tr3, which contains the smallest observed extrachromosomal DNA (approximately 5.5 kb, Figure 1, lane 3) was amplified.

Amplification of wild-type *P. microspora* DNA using the telomeric primer failed to produce a visible product (Figure 4a, lane Wt). However, the same reaction using tr3 total DNA yielded a single amplified product (Figure 4a, lane 1) consistent with the size of the smallest tr3 extrachromosomal DNA visible on Southern blots (Figure 1, lane 3).

In addition, two separate PCR reactions, each using a primer (Hyg1 or Hyg2) complementary to the hygromycin resistance gene in addition to the telomeric primer TE2, yielded products representing a 3.1 kb 5' fragment (Figure 4a, lane 3) and a 2.4 kb 3' fragment (Figure 4a, lane 2) of the target extrachromosomal DNA. This established the position of the hygromycin resistance gene between two blocks of telomeric repeat sequences (Figure 4b). Run-off sequencing of the PCR products (towards the telomeric termini) demonstrated the presence of multiple copies of telomeric repeats on both termini. However, the exact number of repeats on each terminus could not be determined because TE2 has the potential to bind at different positions along the tract of repeats.

*Ban*I restriction digests revealed that the full-length PCR product has restriction fragments in common with pDH33 (Figure 4a, lanes 4 and 5), and demonstrated that the amplified 5' and 3' fragments were in fact fragments of the full-length molecule (data not shown).

Restriction mapping demonstrated the presence of 17 restriction sites in common with pDH33 and failed to reveal a single novel site (Figure 4b). Approximately one-third (1.9 kb) of the amplified extrachromosomal DNA was sequenced, including the important pDH33-telomeric repeat junctions near each terminus (Figure 4c). Sequencing showed that the amplified extrachromosomal DNA is a contiguous, apparently unmodified segment of pDH33 fused directly to terminal telomeric repeats (Figure 4c). The portion of pDH33 present in the extrachromosomal DNA included the hygromycin resistance gene along with its promoter and terminator (Figure 4b) but lacked the bacterial origin of replication and a portion of the ampicillin resistance gene. Thus, this DNA could not have

been obtained by transformation of *E. coli* without additional manipulation.

No fungal sequences other than telomeric repeats were found in the extrachromosomal DNA.

Transformation of wild-type *P. microspora* with the PCR-amplified
5 extrachromosomal DNA produced 10- to 50-fold more transformants per microgram of DNA than did transformation with pDH33, establishing a functional significance for the terminal telomeric repeats present on the *P. microspora* transformant extrachromosomal DNA.

2. Southern Blot Analyses of *P. microspora* Transformants using a Telomeric 10 Probe.

To determine if telomeric repeats are a common feature of extrachromosomal DNAs in different transformants, we probed Southern blots of total DNA with the radio labeled telomeric oligonucleotide TE2. Wild-type total DNA exhibited strong hybridization signal in the region of genomic DNA due to the presence of
15 chromosomal telomeres but revealed no lower molecular weight bands, providing evidence that these do not exist in the untransformed fungus (Fig. 5, lane Wt). However, all extrachromosomal bands in *P. microspora* transformants that hybridized to a radio labeled pDH33 probe (Fig. 1, lanes 1, 3, 4, and 5) and that were not obscured by chromosomal telomere hybridization signal also hybridized to
20 the telomeric probe (Fig. 5, lanes 1, 3, 4, and 5). Thus the telomeric repeats appear to be a common feature of *P. microspora* transformant extrachromosomal DNAs.

Example 8. Sequence Requirements of *P. microspora* Telomerase.

The sequence requirements of *P. microspora* telomerase were further studied by determining additional nucleotide sequences at the pDH33-telomeric repeat
25 junctions near the 5' and 3' termini of the PCR amplified full-length extrachromosomal DNA from transformant tr3, using the protocol as set forth in Experiment 7. As shown by the following examples, the sequence requirements of *P. microspora* telomerase are minimal:

30 10H2T2
taaagcagggaagtatgcgcTTAGGGTTAGGTTTTTAGGGTTAGCTTA(G₃T₂A)_n

(SEQ ID. NO: 3)

15H2T2 acccgatcgtgagcatcct**AGCTTAGGGTTATTA**(G₃T₂A)_n

(SEQ ID. NO: 4)

5

27R tgcatggagccgggcccact**TAGGGTTAGCTTA**(G₃T₂A)_n

(SEQ ID. NO: 5)

21TE2-

10 3315L gtgccggggatcctaggta(G₃T₂A)_n (SEQ ID. NO: 6)

27L tggagccggtgagcgtgggt(TA(G₃T₂A)_n (SEQ ID. NO: 7)

15

16e

tcgatgggggagtatggggg**TAGCTTAGGGTTAGGTTAGCTTA**(G₃T₂A)_n

(SEQ ID. NO: 8)

18.17e ggggagtatgggggagtacc**TAGGGTTAGCTTA**(G₃T₂A)_n

20

(SEQ ID. NO: 9)

21TE2-

1153R gcgacggacgcactgacggt**TAGGG**(G₃T₂A)_n (SEQ ID. NO: 10)

25

Notes:

- (1). Lower case letters are pDH33 nucleotides.
- (2). Upper case letters denote nucleotides which were added *de novo* to pDH33
- (3). Underlined nucleotides are in common with pDH33 and the two telomeric repeats (origin uncertain).

30

- (4). Enzymatic methods have demonstrated that all of the

telomeric repeats added by *P. microspora* telomerase are terminally added (data not provided).

Example 9. Taxol Production Via Generation of Extrachromosomal DNAs in *P. microspora*.

5

The procedures outlined in Examples 1 through 7 are repeated except that the hygromycin resistance gene is replaced with the gene coding for taxadiene synthase. Taxol production of the transformed and non-transformed *P. microspora* cells is compared. The transformed cells display a higher production level of taxol and taxol-related compounds than the non-transformed cells.

10

Example 10. Introduction of Extrachromosomal DNAs into Yew Plants.

The extrachromosomal DNAs coding for taxadiene synthase are extracted from the transformed *P. microspora* cells produced in Example 9 and are used to transform somatic embryos derived from explants of the genus *T. brevifolia*. Taxol and taxol precursors are produced from *in vitro* propagated cultures of somatic embryos derived from the transformed and non-transformed explants. The taxane and taxane-related compounds are produced at a higher level in the transformed explants than they are produced in the non-transformed explants.

15

Example 11. Introduction of Extrachromosomal DNAs into *Taxomyces andreanae*.

20

The extrachromosomal DNAs coding for taxadiene synthase are extracted from the transformed *P. microspora* cells produced in Example 9 and are used to transform cells of the fungus *T. andreanae*. Taxol and taxol precursors are produced from *in vitro* propagated cultures of the transformed and non-transformed fungus. The taxane and taxane-related compounds are produced at a higher level in the transformed fungus than they are produced in the non-transformed fungus.

25

Example 12. "TRAP" Assay for Detection of Telomerase.

30

If the *de novo* addition of telomeres by *P. microspora* is by telomerase activity, then one would expect that telomerase activity in protoplasts (the entities typically transformed) would be detectable using the commonly employed TRAP

assay method. For a detailed description of the TRAP assay, *See*, for example, U.S. Patent No. 5,629,154; Piatyszek, M. A. et al. (1995); and Kim, N. W. *et al.*, each of which is incorporated by reference in their entirety herein.

5 In the TRAP assay, cell extract is mixed with an oligonucleotide which acts as a substrate for telomerase in the extract. Telomerase extends the oligonucleotide in six nucleotide increments. The products of this reaction are then PCR amplified with the original substrate nucleotide, and a second oligonucleotide complementary to the telomeric repeats added by telomerase.

10 Using this method, we positively detected telomerase activity in a cell free extract of *P. microspora* consisting of lysed protoplasts, and all other components the same as the published TRAP assay components (see references cited above for details). The protoplasts were prepared using the same methods as those used to prepare protoplasts for transformation. In the TRAP assays we carried out, the telomerase activity in *P. microspora* protoplasts was heat sensitive, and RNAase
15 sensitive, providing evidence that the positive TRAP assay is due to the presence of a ribonucleoprotein such as telomerase.

The foregoing detailed description has been given for clearness of understanding only and no unnecessary limitations should be understood therefrom
20 as modifications will be obvious to those skilled in the art.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such
25 departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

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30

WHAT IS CLAIMED IS:

1. A method of adding one or more telomeric repeats to DNA which codes for a product which changes the level of taxol production wherein the method comprises
5 introducing the DNA into a *Pestalotiopsis* cell.

2. A method of generating extrachromosomal DNA comprising introducing DNA into a *Pestalotiopsis* cell wherein the DNA codes for a product which changes the level of taxol production.

3. A method of generating a replicable nucleic acid element comprising introducing DNA into a *Pestalotiopsis* cell wherein the DNA codes for a product which changes the level of taxol production.

4. A method of transformation wherein the method comprises:
a) introducing DNA which codes for a product which changes the level of taxol production into a *Pestalotiopsis* cell;
b) permitting one or more telomeric repeats to be added to the DNA to produce extrachromosomal DNA;
c) extracting the extrachromosomal DNA from the transformed
20 *Pestalotiopsis* cell; and
d) introducing the extracted extrachromosomal DNA into a second cell.

5. The method of claim 1, 2, 3 or 4 wherein the DNA has at least 80% sequence
25 similarity to *Pestalotiopsis* DNA.

6. The method of claim 1, 2, 3 or 4 wherein the DNA is not *Pestalotiopsis* DNA.

7. The method of claims 1, 2, 3 and 4 wherein the DNA codes for enzymes selected from the group consisting of taxadiene synthase, taxadiene-5-hydroxylase

and acetyl-coenzyme A.

8. The method of claim 1, 2 or 3 wherein the method further comprises selecting the cell transformed by the introduction of the DNA.

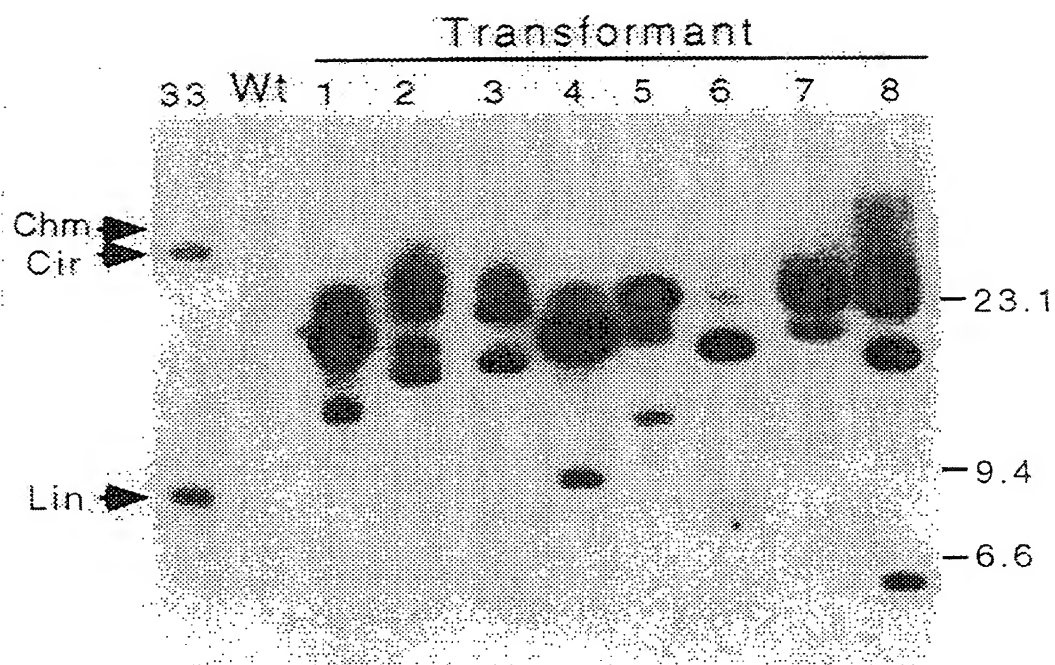
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9. The method of claim 4 wherein the second cell is a eukaryotic cell or a prokaryotic cell.

10. The method of claim 4 wherein the second cell is a *Pestalotiopsis* cell.

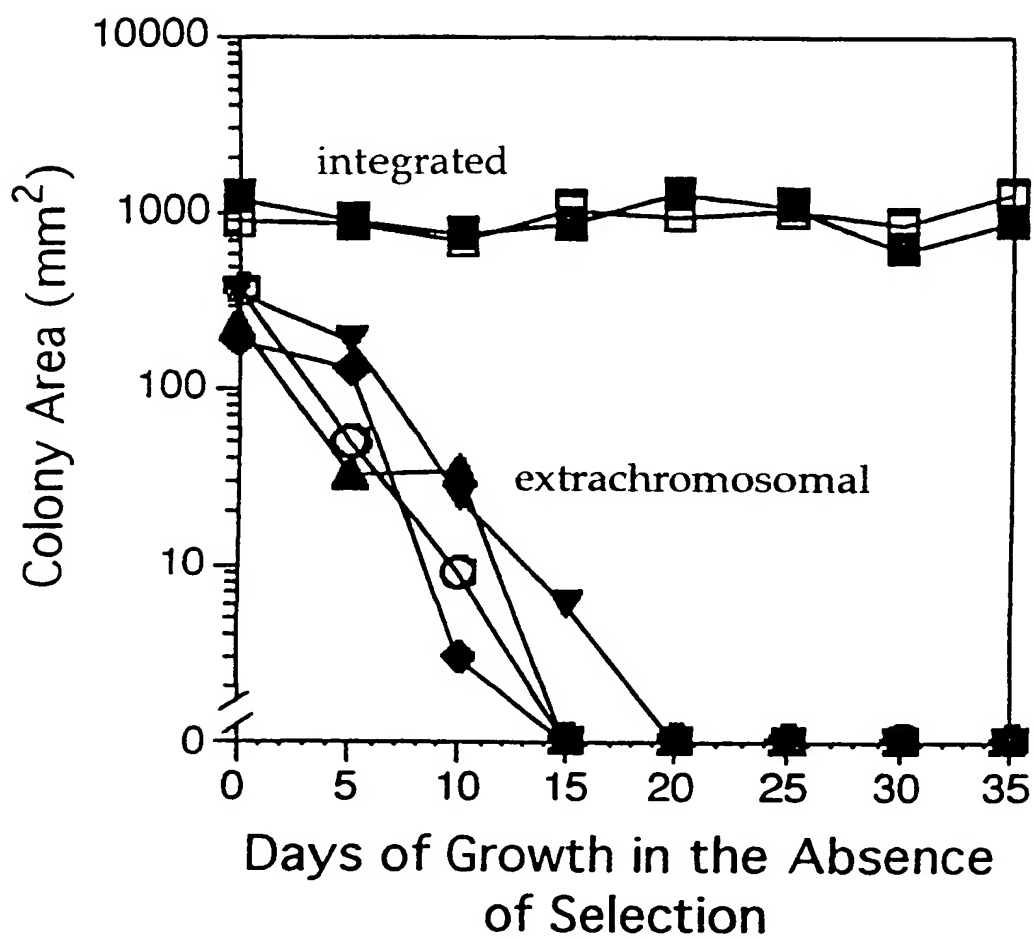
10

11. An artificial chromosome comprising a *Pestalotiopsis* telomerase or *Pestalotiopsis* telomerase subunit and DNA which codes for an enzyme selected from the group consisting of taxadiene synthase, taxadiene-5-hydroxylase and acetyl-coenzyme A.

FIG. 1**BEST AVAILABLE COPY**

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FIG. 2a



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FIG. 2b

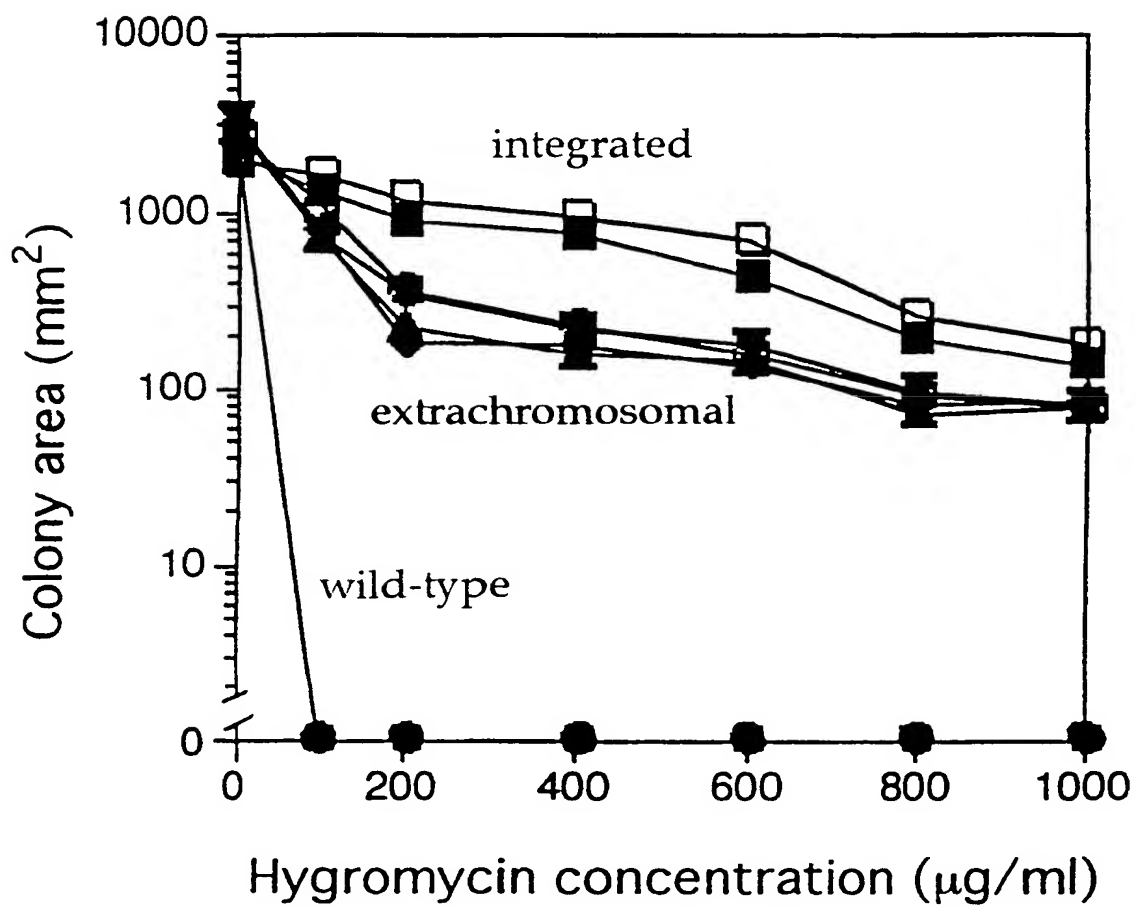


FIG. 3a



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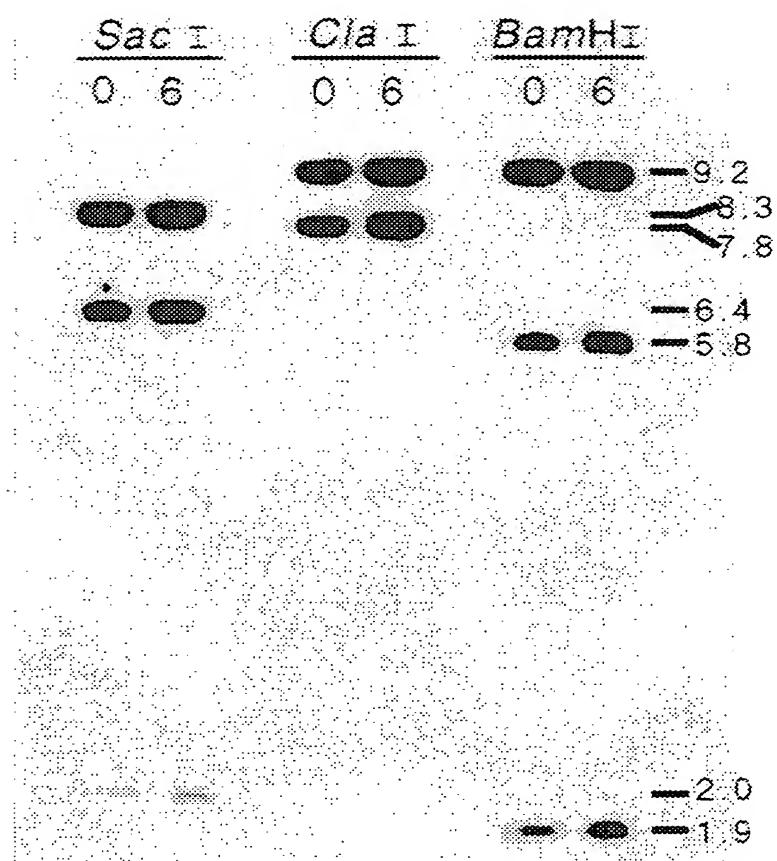
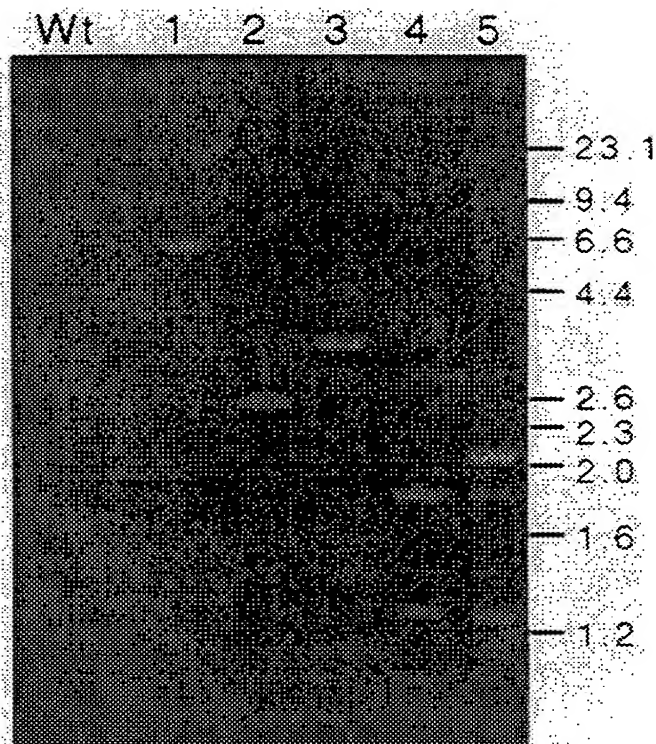
FIG. 3b**BEST AVAILABLE COPY**

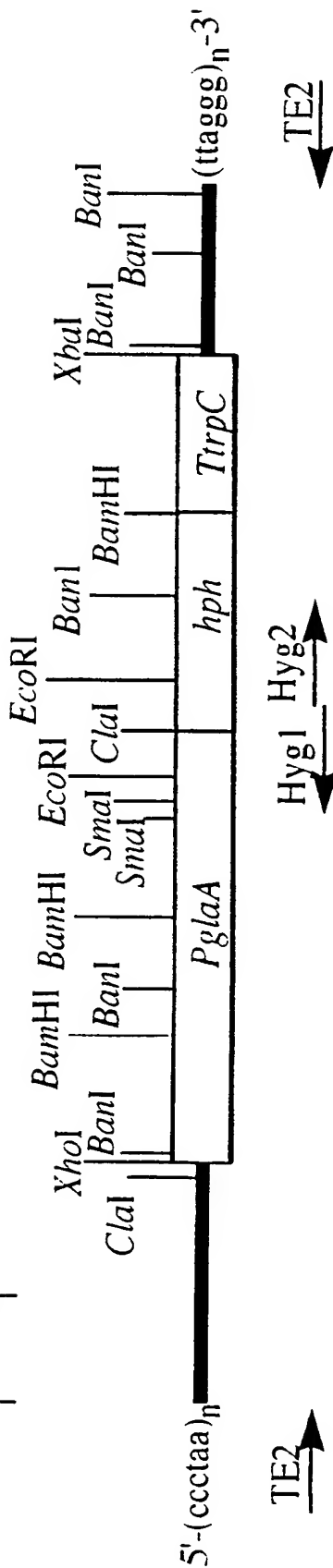
FIG. 4a



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FIG. 4b

500bp



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FIG. 4c

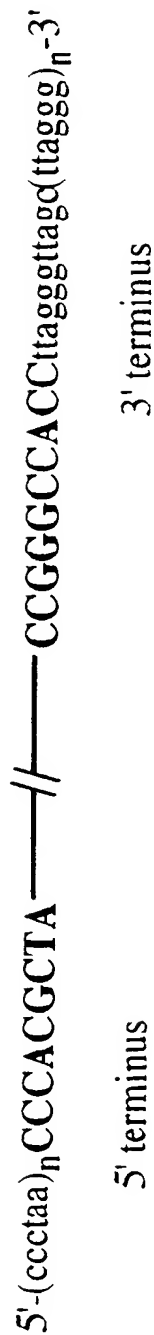
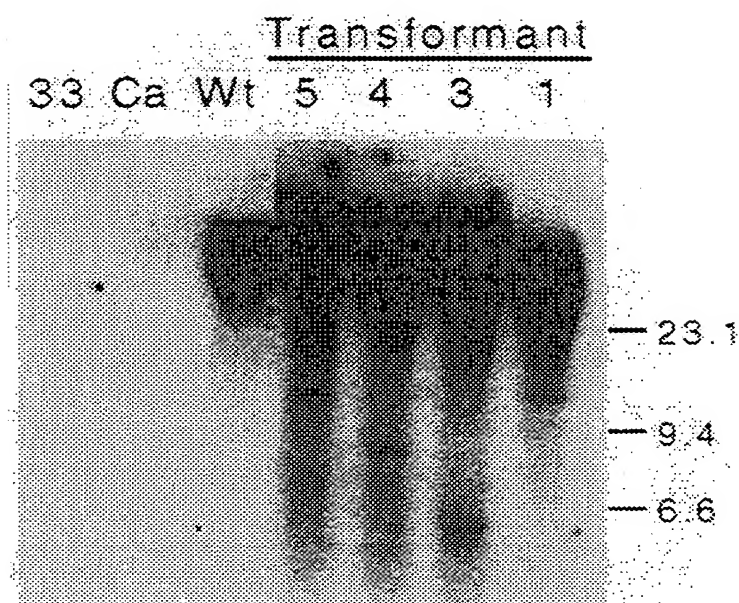


FIG. 5**BEST AVAILABLE COPY**

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/15135

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|--|--|-----------------------|
| A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N15/10 C12N9/12 //C12N9/90,(C12N9/12,C12R1:645) | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | |
| B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 C12N C12P | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | |
| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| P,Y | LONG D.M. ET AL.: "In vivo addition of telomeric repeats to foreign DNA generates extrachromosomal DNAs in the taxol-producing fungus Pestalotiopsis microspora" FUNGAL GENET. BIOL., vol. 24, no. 3, August 1998 (1998-08), pages 335-344, XP000856850 the whole document | 1-11 |
| Y | WO 97 38571 A (UNIV WASHINGTON) 23 October 1997 (1997-10-23) abstract page 3, line 8 - line 12 claims 28-30 | 1-11 |
| --- -/-- | | |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. | | |
| <input checked="" type="checkbox"/> Patent family members are listed in annex. | | |
| * Special categories of cited documents : <div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | |
| Date of the actual completion of the international search | Date of mailing of the international search report | |
| 1 December 1999 | 15/12/1999 | |
| Name and mailing address of the ISA | Authorized officer | |
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Galli, I | |

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International Application No

PCT/US 99/15135

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|----------|--|-----------------------|
| A | <p>National Science Foundation, Grant No. 9724999, 1.7.1997-30.6.1998 Long D.M. & Strobel G.A., "SGER: efficient extrachromosomal replication of exogenous DNA by a filamentous fungus". http://fundedresearch.cos.com/cgi-bin/NSF/ getRec?9724999 XP002124429 cited in the application abstract</p> <p style="text-align: center;">---</p> | 1-11 |
| A | <p>STROBEL ET AL: "Taxol from Pestalotiopsis microspora, an endophytic fungus of Taxus wallachiana" MICROBIOLOGY, vol. 142, no. 142, 1996, page 435-440-440 XP002103985 ISSN: 1350-0872 cited in the application the whole document</p> <p style="text-align: center;">---</p> | 1-11 |
| A | <p>JAVERZAT J.-P. ET AL.: "Isolation of telomeric DNA from the filamentous fungus Podospora anserina and construction of a self-replicating linear plasmid showing high transformation frequency" NUCL. ACIDS RES., vol. 21, no. 3, 1993, pages 497-504, XP002124428 the whole document</p> <p style="text-align: center;">-----</p> | 1-11 |

INTERNATIONAL SEARCH REPORT

information on patent family members

Interr. 1st Application No

PCT/US 99/15135

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO 9738571 A | 23-10-1997 | AU 710405 B | 16-09-1999 |
| | | AU 2922597 A | 07-11-1997 |
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| ----- | | | |